IMMUNE MECHANISMS IN ATHEROSCLEROSIS

Focus on the Role of the Complement System

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ACADEMIC DISSERTATION

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To my family

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I. Oksjoki R, Jarva H, Kovanen PT, Laine P, Meri S, Pentikäinen MO. Association between complement factor H and proteoglycans in early human coronary atherosclerotic lesions; Implications for local regulation of complement activation. *Arterioscler Thromb Vasc Biol.* 2003, 23:630-636.

II. Oksjoki R, Kovanen PT, Mäyränpää MI, Laine P, Blom A, Meri S, Pentikäinen MO. Complement regulation in human atherosclerotic lesions; Immunohistochemical evidence that C4b-binding protein regulates the classical complement pathway and that C5b-9 is formed via the alternative complement pathway. *Atherosclerosis. 2006, In press.*

III. Oksjoki R*, Laine P*, Helske S, Vehmaan-Kreula P, Mäyränpää MI, Gasque P, Kovanen PT, Pentikäinen MO. Receptors for the anaphylatoxins C3aR and C5aR are expressed in human atherosclerotic coronary plaques. *Submitted.*
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IV. Oksjoki R, Lindstedt KA, Jansson B, Kovanen PT, Pentikäinen MO. OxLDL-IgG immune complexes induce survival of human monocytes. *Arterioscler Thromb Vasc Biol.* 2006, 26:576-583.

ABBREVIATIONS

ASP	acylation-stimulating protein
AHA	American Heart Association
AMI	acute myocardial infarction
β2-GP1	β2-glycoprotein I
C4bp	C4b-binding protein
CAD	coronary artery disease
CMV	cytomegalovirus
CVD	cardiovascular disease
CVID	common variable immunodeficiency
DAF	decay-accelerating factor
EBV	Epstein-Barr virus
E-LDL	enzymatically modified LDL
GM-CSF	granulocyte-macrophage colony-stimulating factor
4-HNE	4-hydroxynonenal
HSP	heat shock protein
HSV	herpes simplex virus
IC	immune complex
IEL	internal elastic lamina
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
IMT	intima-media thickness
KLH	keyhole limpet hemocyanin
LFA-1	lymphocyte function-associated antigen-1
MAC	membrane-attack complex
MASP	mannose-binding lectin-associated serine proteases
MBL	mannose-binding lectin
MCP	membrane cofactor protein
MCP-1	monocyte chemotactic protein-1
mCRP	modified CRP
M-CSF	monocyte colony-stimulating factor
MDA	malondialdehyde
mmLDL	minimally oxidized LDL
NHS	normal human serum
NIDDM	non-insulin-dependent diabetes mellitus
NK cell	natural killer cell
NKT cell	natural killer T cell
oxLDL	oxidized low density lipoprotein
PAMP	pathogen-associated molecular pattern
PC	phosphocholine
PLA_2	phospholipase A_2
PMA	phorbol 12-myristate 13-acetate
POVPC	1-palmitoyl-2-(5'-oxovaleroyl)-sn-glycero-3-phosphocholine
PRR	pattern recognition receptor
PTX	pentraxin
SCR	short consensus repeat
SLE	systemic lupus erythematosus
SMC	smooth muscle cell
SR-A	scavenger receptor A
TBARS	thiobarbituric acid-reactive substances
TCR	T cell receptor
TGF-β	transforming growth factor-β
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
Treg	regulatory T cell
VALT	vascular-associated lymphoid tissue
VLA-4	very late antigen-4
VCAM-1	vascular cell adhesion molecule-1
WHHL	Watanabe Heritable Hyperlipidemic (rabbits)

ABSTRACT

Atherosclerosis is an inflammatory disease characterized by accumulation of lipids in the inner layer of the arterial wall. During atherogenesis, various structures that are recognized as non-self by the immune system, such as modified lipoproteins, are deposited in the arterial wall. Accordingly, atherosclerotic lesions and blood of humans and animals with atherosclerotic lesions show signs of activation of both innate and adaptive immune responses. Although immune attack is initially a self-protective reaction, which is meant to destroy or remove harmful agents, a chronic inflammatory state in the arterial wall accelerates atherosclerosis. Indeed, various modulations of the immune system of atherosclerosis-prone animals have provided us with convincing evidence that immunological mechanisms play an important role in the pathogenesis of atherosclerosis.

This thesis focuses on the role of complement system, a player of the innate immunity, in atherosclerosis. Complement activation via any of the three different pathways (classical, alternative, lectin) proceeds as a self-amplifying cascade, which leads to the generation of opsonins, anaphylatoxins C3a and C5a, and terminal membrane-attack complex (MAC, C5b-9), all of which regulate the inflammatory response and act in concert to destroy their target structures. To prevent uncontrolled complement activation or its attack against normal host cells, complement needs to be under strict control by regulatory proteins. The complement system has been shown to be activated in atherosclerotic lesions, modified lipoproteins and immune complexes containing oxLDL, for instance, being its activators.

First, we investigated the presence and role of complement regulators in human atherosclerotic lesions. We found that inhibitors of the classical and alternative pathways, C4b-binding protein and factor H, respectively, were present in atherosclerotic lesions, where they localized in the superficial proteoglycan-rich layer. In addition, both inhibitors were found to bind to arterial proteoglycans *in vitro*. Immunohistochemical stainings revealed that, in the superficial layer of the intima, complement activation had been limited to the C3 level, whereas in the deeper intimal layers, complement activation had proceeded to the terminal C5b-9 level. We were also able to show that arterial proteoglycans inhibit complement activation *in vitro*. These findings suggested to us that the proteoglycan-rich layer of the arterial intima contains matrix-bound complement inhibitors and forms a protective zone, in which complement activation is restricted to the C3 level. Thus, complement activation is regulated in atherosclerotic lesions, and the extracellular matrix is involved in this process.

Next, we studied whether the receptors for the two complement derived effectors, anaphylatoxins C3a and C5a, are expressed in human coronary atherosclerotic lesions. Our results of immunohistochemistry and RT-PCR analysis showed that, in contrast to normal intima, C3aR and C5aR were highly expressed in atherosclerotic lesions. In atherosclerotic plaques, the principal cells expressing both C3aR and C5aR were macrophages. Moreover, T cells expressed C5aR, and a small fraction of them also expressed C3aR, mast cells expressed C5aR, whereas endothelial cells and subendothelial smooth muscle cells expressed both C3aR and C5aR. These results suggested that intimal cells can respond to and become activated by complement-derived anaphylatoxins.

Finally, we wanted to learn, whether oxLDL-IgG immune complexes, activators of the classical complement pathway, could have direct cellular effects in atherogenesis. Thus, we tested whether oxLDL-IgG immune complexes affect the survival of human monocytes, the precursors of macrophages, which are the most abundant inflammatory cell type in atherosclerotic lesions. We found that OxLDL-IgG immune complexes, in addition to transforming monocytes into foam cells, promoted their survival by decreasing their spontaneous apoptosis. This effect was mediated by cross-linking Fcγ receptors with ensuing activation of Akt-dependent survival signaling. Our finding revealed a novel mechanism by which oxLDL-IgG immune complexes can directly affect the accumulation of monocyte-macrophages in human atherosclerotic lesions and thus play a role in atherogenesis.

I INTRODUCTION

Cardiovascular diseases are the leading cause of death in the Western world, and they are also expected to be the main cause of global death within the next 15 years, since their prevalence is rapidly increasing even in the developing countries. Atherosclerosis (Greek 'athero' = gruel/paste; 'sclerosis' = hardening) is the most common underlying pathologic process leading to cardiovascular morbidity and mortality. It is a slow, complex process characterized by accumulation of lipids and thickening of the inner layer of the arterial wall, which, in its earliest form, appears to be present even at the fetal stage, and evolves slowly over decades. Atherosclerosis of coronary arteries may lead to compromised blood flow and oxygen supply to the myocardium. This may cause clinical symptoms due to chronic narrowing of the lumen (stable angina) or abrupt plaque erosion or rupture with ensuing thrombus formation (unstable angina, myocardial infarction).

Hypercholesterolemia is a major risk factor for this disease, and cholesterol has been shown to be an absolute prequisite for lesion initiation and progression. In 1913, an experimental pathologist named Anitschkow found out that diet-induced hypercholesterolemia in rabbits leads to the generation of vascular lesions resembling human atherosclerosis. Hypercholesterolemia, and other 'traditional risk factors' such as tobacco smoking, hypertension, diabetes, male gender, age, and family history define much of the risk of the disease. However, many newly found 'non-traditional risk factors' such as inflammation are emerging and are considered to be of importance in the pathophysiology of atherosclerosis.

More than a century ago already, Dr. Rudolf Virchow suggested that the generation of an atheroma results from an inflammatory process in the intima, but this view was ignored for decades, while the role of cholesterol dominated the scientific thinking. During the recent decades, however, much attention has been paid to inflammatory and immune mechanisms in the pathogenesis of atherosclerosis, and it has become apparent that these mechanisms play an important role in modulating the course of the disease. The current concept suggests that, during atherogenesis, various antigens, i.e. structures that are recognized as non-self by the immune system, are generated or retained in the intima and trigger an inflammatory and immune response against them. Thus, atherosclerosis has come to be regarded as a chronic inflammatory disease.

The purpose of the present work was to examine the activation of immune mechanisms in atherosclerotic lesions with a specific focus on the complement system and the role of immune complexes containing oxidized LDL.

II LITERATURE REVIEW

1 Pathogenesis of atherosclerosis

1.1 Normal arterial intima

The arterial wall consists of three layers: intima, media and adventia (Fig. 1). Intima is the innermost layer of the arterial wall, separated from the vacular lumen and circulating blood by only a single layer of endothelial cells. The endothelium, residing at the critical interface between blood flow and the arterial wall, functions as a permeability barrier, forms a thromboresistant surface, and attends to the regulation of vascular tone by producing prostacyclin, nitric oxide, and endothelin. The intima is composed of two layers; a superficial proteoglycan-rich layer and a deeper musculoelastic layer. Often, these layers can only be recognized in areas of adaptive intimal thickening (see later). The extracellular matrix of the superficial proteoglycan-rich layer is mainly composed of chondroitin sulfate-rich proteoglycan versican, and to a lesser extent, two smaller leucine-rich dermatan sulfate proteoglycans called decorin and biglycan (reviewed by Williams 2001) The matrix in this area also contains some collagen, mainly types I and III, elastin, and glycoproteins (Stary *et al.* 1992). The main sources of extracellular matrix are rough endoplasmic reticulum-rich smooth muscle cells of synthetic phenotype located scattered in the subendothelial region. In addition, some contractile, myofilament-rich smooth muscle cells are also present. The subendothelial space also contains some macrophages, mast cells, and T cells (Kaartinen *et al.* 1994a). The deeper musculoelastic layer contains a large number of myofilament-rich, contractile smooth muscle cells, which are organized as close layers. The musculoelastic layer contains more collagen than the proteoglycan-rich layer and is rich in elastic fibers. The intima is separated from the middle layer of the vessel wall, the media, by continuous elastic lamina, called internal elastic lamina (IEL).

Even without any signs of atherosclerosis, the intima of human epicardial coronary arteries is thickened in response to changes in physiological factors such as increased tension or disturbance of shear stress in the vessel wall. These adaptive intimal thickenings can be either diffuse or eccentric. In general, intimal thickening in coronary arteries is diffuse, and eccentric intimal thickening typically only occurs at the branching points of the arteries, involving half of the vessel circumference. Intimal thickening starts during fetal life, and all individuals have such thickenings at the time of birth (Stary *et al.* 1992).

1.2 Morphological changes during atherogenesis

The same mechanical forces that give rise to intimal thickenings also enhance the influx of plasma lipoproteins and other circulating molecules into the arterial intima. Thus, lesions evolve first and more rapidly in atherosclerosis-prone areas with eccentric intimal thickening of the vessel wall (Stary 1989). Based on the morphological changes observed in the vessel wall, especially in the intima, during the pathogenesis of atherosclerosis, lesions have been classified into types I-VIII by American Heart Association (AHA) (Fig. 1). Initial type I lesions are characterized by an increased number of macrophages, organized as small groups, which have been transformed into foam cells upon the uptake of lipids (Stary *et al.* 1994). When lipid-filled macrophages and smooth-muscle cells become more numerous and are organized in layers, the lesions are classified as type II lesions. In areas of thin intima, these

changes may be visible to the naked eye as flat, yellow-colored streaks in the inner surface of arteries, and they are called fatty streaks. In contrast, when colocalized with adaptive intimal thickening, foam cells locate deeper in the intima, in which case the changes might be invisible to the naked eye. In type II coronary lesions, the numbers of macrophages, T lymphocytes, and mast cells are increased (Kaartinen *et al.* 1994a). These minimal changes do not disrupt the normal structure of the arterial wall, presumably stabilize without any additional stimulus, and are clinically silent. Early lesions (types I and II) are typically present in children, but are also found in adults, especially in areas that are resistant to atherosclerosis. Type II lesions that locate in highly-susceptible, atherosclerosis-prone areas may progress to type III lesions, which are intermediate lesions between early and advanced atherosclerotic lesions. These lesions, which are present from young adulthood onward, are characterized by the accumulation of lipids as small separate pools in the extracellular space between smooth muscle cells, disrupting the space between closely adjoining smooth muscle cells in the intima.

With the progression of atherosclerosis, typically from the third decade of life onward, the small lipid pools of type III lesions confluence into a larger, more disruptive extracellular lipid core, a hallmark type IV lesion (atheroma). The lipid derives not only from the free lipid droplets entered from plasma, but also from the remnants of dying foam cells. The lipid core may also contain cholesterol crystals and calcium particles. The intima overlying the core is usually of the same thickness as in type I and II lesions and contains macrophages and smooth muscle cells with or without intracellular lipids as well as T lymphocytes and mast cells. These lesions clearly thicken the arterial wall, but they may not diminish the diameter of the vascular lumen, since the vessel wall may first slightly expand outward, showing compensatory remodeling. In response to tissue disruption, intimal smooth-muscle cells start to proliferate and form new connective tissue matrix, especially collagen. This leads to thickening of the superficial intima overlying the lipid core, and consequently, in the formation of a fibrous cap, which separates the lipid core from the vessel lumen (type V lesion, fibroatheroma) (Stary *et al.* 1995). These new layers oppose the outward expansion, and may narrow down the vessel lumen. A report by Virmani and coworkers (Virmani *et al.* 2000) divided type IV-V atheromas based on the thickness of the connective tissue layer covering the lipid core, i.e. the fibrous cap. “The thin fibrous cap atheromas” (cap <65 μm thick) are characterized by a loss of smooth muscle cells and extracellular matrix and typically have a large underlying lipid core and are most likely to rupture.

Lesions complicated by a surface defect (rupture, erosion, calcified nodule) and thrombosis are classified as type VI lesions. The thrombus may cause clinical symptoms, such as myocardial infarction, unstable angina pectoris, or sudden coronary death, but it may also disappear without symptoms. Data suggest that 60% of sudden coronary deaths result from a rupture and subsequent thrombus formation, whereas erosions are the underlying cause of 40% of thrombotic sudden coronary deaths (van der Wal *et al.* 1994). In plaque rupture, the fibrous cap is disrupted, usually at its weakest point, i.e. the shoulder region, and the luminal thrombus that develops is in communication with the lipid-rich necrotic core. The susceptibility of lesions to rupture is greatly influenced by the composition and structure of the underlying lesion. Ruptured lesions typically have an extensive necrotic core, a thin fibrous cap, scant smooth muscle cells, and large infiltration of inflammatory cells, which may further weaken the cap by producing toxic substances and matrix-degrading enzymes (Naghavi *et al.* 2003; van der Wal *et al.* 1994). In these plaques, moreover, *vasa vasorum* are typically present within the plaque and adventitia. These neovessels may contribute to destabilization of the plaque, since intraplaque hemorrhage has been shown to increase

significantly the size of the necrotic core (Kolodgie *et al.* 2003). Plaque erosion is characterized by superficial erosions, in which typically only the endothelial cell layer is detached. The intima underlying the erosion shows various compositions; some have characteristics of type III lesions, some contain a lipid core, and yet some others are entirely fibrous. In addition, especially in younger patients and women, the intima underlying erosions usually contains smooth muscle cells and proteoglycans. The results regarding the role of inflammatory process in erosion are controversial (Farb *et al.* 1996; van der Wal *et al.* 1994). The least frequent cause of thrombotic events, calcific nodule, refers to disruption of the endothelium/thin fibrous cap overlying a heavily calcified plaque.

Histological evidence suggests that complicating episodes may occur in succession. During recovery, fissures are sealed, the thrombus is organized as part of the growing plaque, and endothelial cells overgrow the thrombus at the lumen. This "healing" process is a typical mechanism for plaque growth and may also lead to the formation of advanced plaques, in which calcification (type VII lesion) or fibrous tissue (type VIII lesion) predominates. Regression or change of lipid content in the lesion types IV-V may also result in type VII and VIII lesion.

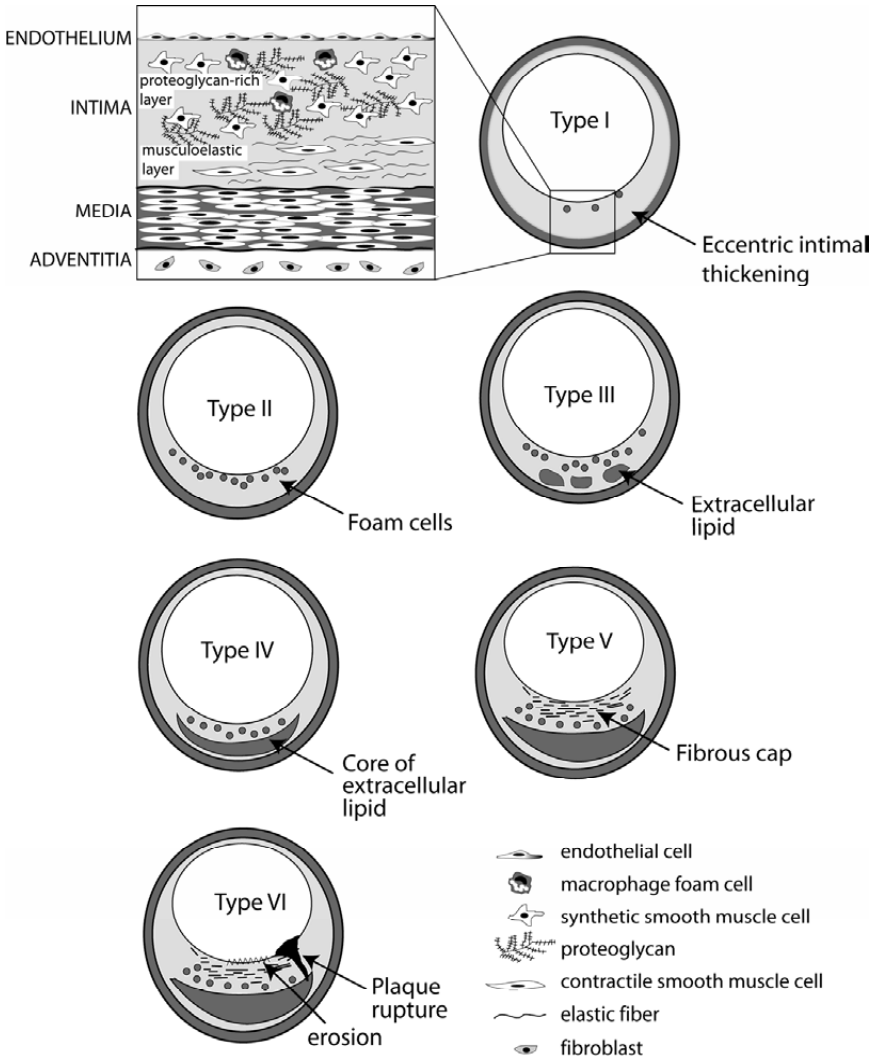

Figure 1. Structure of the arterial wall and histolocigal classification of atherosclerotic lesions (according to the American Heart Association). Adapted from Stary *et al.* (1995).

1.3 Retention and modification of LDL during atherogenesis

Infiltration and accumulation of atherogenic lipoproteins in the arterial intima constitutes a central pathogenic process in atherogenesis (reviewed by Williams & Tabas 1995). General view suggests that accumulation of LDL in atherosclerosis-prone areas of the arterial tree is not dependent on increased permeability of the endothelium or an increased rate of lipoprotein entry into the arterial wall, but it results mainly from increased retention of lipoproteins in the intima in these disease-prone areas (Schwenke & Carew 1989). Upon entering the arterial intima from circulation, LDL particles become entrapped in the tight extracellular matrix network of the intima and also bind to the components of the matrix, especially to proteoglycans. Interestingly, *in vitro* studies have shown that binding of LDL to proteoglycans is increased when proteoglycans originate from atherosclerotic-prone areas of the arterial tree (Cardoso & Mourao 1994). This binding is dependent on the interaction between positively charged lysine and arginine residues of apoB-100 of LDL and negatively charged glycosaminoglycans of proteoglycans. More specifically, Boren and coworkers showed that, when lysine3363 in apoB was replaced by glutamic acid, the binding of LDL to proteoglycans was severely defective (Boren *et al.* 1998). Moreover, transgenic mice expressing the mutated apoB-100 that does not bind to proteoglycans show strongly retarded development of atherosclerosis (Skålen *et al.* 2002), which clearly demonstrates the importance of LDL-proteoglycan interaction and lipoprotein retention in early atherogenesis. In addition to this direct binding of apoB to proteoglycans, LDL may be linked to PGs by molecules such as LPL, which is locally produced by e.g. macrophages (reviewed by Pentikäinen et al. 2002). Entrapment and retention of LDL by the extracellular matrix prolongs its residence in the intima and predisposes it to modifications by enzymes and agents secreted by intimal cells (Pentikäinen *et al.* 1997). In vitro studies have shown that various modifications of LDL (oxidation, proteolysis, and phospholipolysis) can disrupt the surface structure of LDL particles and lead to aggregation and fusion of lipoprotein particles (Pentikäinen *et al.* 1996). Such modification-induced changes appear to be required for the accumulation of LDL-derived lipids both extra- and intracellularly in the arterial intima. Indeed, the arterial intima has been shown to contain extracellular lipid droplets and pools of lipids, and when extracted they possess qualities that suggest that they derive from plasma LDL particles that have undergone modifications of various types (reviewed by Öörni *et al.* 2000). Furthermore, modified lipoproteins have been detected in the arterial wall by immunohistochemical methods (Torzewski *et al.* 1998b; Jurgens *et al.* 1993). Modifications of LDL also promote its intracellular accumulation, since macrophages and smooth muscle cells recognize and take up modified LDL via various scavenger receptors. In contrast to the uptake of native LDL, cholesterol uptake via scavenger receptors is not subject to negative feedback regulation, and it thus leads to uncontrolled endocytosis and transformation of cells into lipid-filled foam cells (Goldstein *et al.* 1979).

Of the different modifications of LDL, oxidation was long considered the single most important modification with regard to atherogenesis. As described above, oxidation of LDL promotes the accumulation of LDL both extra- and intracellularly, but it also changes LDL into immunogenic and pro-inflammatory particles (reviewed by Hörkkö *et al.* 2000). In the oxidative process of LDL, both lipids and apoB-100 are oxidized. Oxygen radicals attack unsaturated fatty acids residues in lipoprotein, notably in surface phospholipids, and this lipid peroxidation leads to the release of aldehyde fragments (malondialdehyde; MDA and 4-hydroxynonenal; 4-HNE), which may react with apoB-100 in LDL by binding to lysine residues. In addition, reactive oxidation products derived from phospholipids such as POVPC (1-palmitoyl-2-(5’-oxovaleroyl)-sn-glycero-phosphocholine) can form covalent adducts with

apoB-100 of LDL (reviewed by Hörkkö *et al.* 2000). Circulating LDL, despite the potent antioxidant capacity of plasma, has been shown to contain oxidized epitopes (Palinski *et al.* 1996; Tsimikas *et al.* 2005). While the origin of these circulating oxidized lipoprotein particles remains unclear, convincing evidence indicates that oxidative modification of LDL occurs in the microenvironments of atherosclerotic arterial wall, where LDL is no longer efficiently protected by antioxidants of plasma. Indeed, LDL isolated from atherosclerotic lesions, but not from normal intima, shows signs of oxidation, i.e. increased electrophoretic mobility, and reacts with the antibody against MDA-modified LDL (Ylä-Herttuala *et al.* 1989). In addition, immunohistochemical methods have shown oxidation specific epitopes in LDL in atherosclerotic lesions (Palinski *et al.* 1996; Palinski *et al.* 1989; Jurgens *et al.* 1993). Regarding the mechanisms of LDL oxidation *in vivo*, various alternatives have been proposed, and it is known that various cell types present in atherosclerotic lesions are able to mediate lipid oxidation *in vitro* (reviewed by Steinberg 1997). While transition metal ions (Cu^{2+} or Fe^{2+}) have been suggested to play only a minor role in the oxidation of LDL in atherosclerotic lesions, more evidence exists for the role of 15-lipoxygenase, myeloperoxidase, and reactive nitrogen species in this process (reviewed by Heinecke 1997). All these agents are capable of causing LDL oxidation *in vitro*, and they are present in atherosclerotic, but not in normal, arterial wall. In addition, markers of oxidation by 15-lipoxygenase, myeloperoxidase and reactive nitrogen species have been detected in atherosclerotic lesions, and LDL isolated from atherosclerotic lesions has been shown to contain signs of oxidation by these agents (Heinecke 1997).

In addition to oxidative enzymes, atherosclerotic lesions also contain various proteolytic and lipolytic enzymes capable of non-oxidatively modifying LDL either at neutral or acidic pH. Proteolytic modifications may degrade the apoB-100 moiety of LDL, whereas lipases can hydrolyze phospholipids, triglycerides and cholesterol esters of LDL. Both proteolysis and phospholipolysis of LDL may lead to changes in the surface structure of LDL, which, similarly to oxidation, may lead to aggregation and fusion of lipoprotein particles (reviewed by Öörni *et al.* 2000). Proteases present in atherosclerotic lesions include mast cell-derived tryptase and chymase (Kaartinen *et al.* 1994b), plasma-derived plasmin (Grainger *et al.* 1994), kallikrein (Cerf *et al.* 1999), thrombin (Smith *et al.* 1996), various matrix metalloproteinases (reviewed by Garcia-Touchard *et al.* 2005), and lysosomal proteases including cathepsins G, S, K, F and D (Legedz *et al.* 2004; Sukhova *et al.* 1998; Öörni *et al.* 2004; Hakala *et al.* 2003). In addition, various lipases such as acid sphingomyelinase (Marathe *et al.* 1999), phospholipase A_2 (PLA_2)(Romano *et al.* 1998), carboxyl ester lipase (Li & Hui 1998), and lysosomal acid lipase (Hakala *et al.* 2003) are present in the intima. To support their role in the modification of LDL *in vivo*, LDL extracted from arterial intima shows signs which suggest modification by both proteases and lipases (Öörni *et al.* 2000). In addition to these changes in lesion-derived LDL, enzymatically modified LDL has also been detected in atherosclerotic lesions by immunohistochemical methods. In these experiments, Torzewski and coworkers created an antibody against LDL modified with trypsin, neuraminidase, and cholesterol esterase (E-LDL), a modification that creates LDL with properties akin to lipids extracted from atherosclerotic lesions (Bhakdi *et al.* 1995), and found LDL containing such modified epitopes in human atherosclerotic lesions (Torzewski *et al.* 1998b).

2 Immune mechanisms in atherosclerosis

There is potent evidence to indicate that the immune system plays an important role in atherogenesis and its complications. Very soon after the introduction of an atherogenic diet, the endothelium of rabbit vessel wall shows attached leukocytes (Poole & Florey 1958), and the atherosclerotic lesions of animal models and humans have been shown to contain infiltrates of immune cells, particularly macrophages and T cells (Jonasson *et al.* 1986). This accumulation of immune cells during early atherogenesis suggests that immune activation toward plaque material plays a role in atherogenesis. In addition, the sites of plaque rupture contain activated macrophages, T cells, mast cells, and neutrophils (Kovanen *et al.* 1995; van der Wal *et al.* 1994; Naruko *et al.* 2002), suggesting that activation of the immune system also has implications for the later stages of the disease. In addition to inflammatory cells, atherosclerotic lesions contain other signs of immune activation, such as activation products of the complement system (Vlaicu *et al.* 1985a). Furthermore, circulating autoantibodies against plaque material and their correlation with cardiovascular diseases strongly support the role of immune activation in atherogenesis (reviewed by Nilsson & Kovanen 2004). Finally, the most convincing evidence derives from experimental studies demonstrating that specific immune-based interventions can influence lesion progression in animal models.

Both innate and adaptive immune systems are activated in atherosclerotic lesions and act in concert to destroy foreign or altered self structures. Despite the established division into innate and adaptive immunity, it is worth remembering that there is a vast amount of cross-talk between these systems and simultaneous activation of both cellular and humoral types of response. Although, initially, an immune attack is regarded as a self-protective reaction meant to destroy the harmful agents, chronic inflammation in the arterial wall appears to be detrimental and self-perpetuating inflammatory state leading to aggravation of atherosclerosis and even rupture or erosion of plaque.

2.1 What are the triggers of the immune response in atherosclerotic plaques?

The nature of the process and agents that elicit a localized inflammatory and immune response in atherosclerotic lesions is not totally clear. It is likely that, once lesion formation has begun, various antigens are generated within the atherosclerotic lesion. Indeed, Paulsson and coworkers showed that atherosclerotic lesions of apoE$^{-/-}$ mice show preferential expression of TCR-variable regions, suggesting oligoclonal rather than polyclonal expansion of T cells and indicating that a limited number of antigens induce an immune response in atherosclerotic plaques (Paulsson *et al.* 2000). Regarding possible antigens, modified lipoproteins are obvious candidates. In addition, various other candidates, including endogenous agents such as β2-glycoprotein 1 and heat shock proteins, as well as antigens derived from exogenous pathogens have been proposed.

2.1.1 Oxidized LDL

The most extensive data obtained so far support a role for oxLDL as a trigger of an immune response in atherosclerotic lesions (reviewed by Hörkkö *et al.* 2000). When LDL accumulates extracellularly in arterial intima, it is predisposed to oxidative modification (see section *Retention and modification of LDL during atherogenesis*) leading to the generation of a variety of highly immunogenic neo-determinants, which can trigger the activation of both

innate and adaptive types of immune responses. Regarding innate immunity, oxLDL has been shown to bind to various pattern recognition receptors (reviewed by Miller *et al.* 2003a). In addition, oxLDL antibodies cloned from the spleens of hypercholesterolemic apoE$^{-/-}$ mice have been shown to belong to the class of highly conserved natural antibodies of innate immunity, and they immunostain both rabbit and human atherosclerotic lesions (Palinski *et al.* 1996). There is also evidence about the activation of an adaptive immune response against oxLDL. Indeed, 15% of T cells isolated from human plaques and cloned and expanded in culture proliferated in response to oxLDL in a HLA-DR-dependent manner (Stemme *et al.* 1995), and human peripheral T cells have also been shown to become activated by oxLDL (Frostegård *et al.* 1992). Further, the spleens of apoE$^{-/-}$ mice give rise to oxLDL-specific T cell lines that display strong immunity to oxLDL (Caligiuri *et al.* 1999). In addition, autoantibodies against oxLDL have been detected in the plasma of hypercholesterolemic experimental animals, in which their levels correlated with the severity of the atherosclerosis (Palinski *et al.* 1995b). In humans, in contrast, both positive and negative correlations between the levels of oxLDL-autoantibodies and cardiovascular diseases have been reported (see section *OxLDL autoantibodies of adaptive immunity*). Moreover, immunization studies have shown that immunization of atherosclerosis-prone animals with prepararations of oxLDL decreased the size of lesions (see section *Effects of immune system in atherogenesis*). Interestingly, the transfer of purified CD4+ cells from oxLDL-immunized mice to immunodeficient apoE$^{-/-}$ mice aggravated atherosclerosis more than T cells from mice immunized with KLH (Zhou *et al.* 2006).

2.1.2 β2-glycoprotein 1

β2-glycoprotein 1 (β2-GP1) is a highly glycosylated plasma protein, of which about 40% circulates in association with lipoproteins of various classes, and complexes between β2-GP1 and oxLDL have also been described (Kobayashi *et al.* 2003). β2-GP1 binds avidly to negatively charged phospholipids surfaces and is suggested to be a major target for autoimmune type antiphospholipids antibodies (McNeil *et al.* 1990). Furthermore, Hörkkö and coworkers suggested that, analogous to LDL oxidation, some of the epitopes for autoimmune antibodies are generated, when oxidized phospholipids form covalent adducts with associated proteins, such as β2-GP1, and thus form neoepitopes recognized by antiphospholipid antibodies (Hörkkö *et al.* 1997; Hörkkö *et al.* 2001). β2-GP1 is present in human atherosclerotic lesions, where it is mainly found in the subendothelial area colocalized with CD4+ T lymphocytes (George *et al.* 1999a). Studies on experimental animals have shown that immunization of atherosclerosis-prone mice with human β2-GP1 induces both cellular and humoral immune responses against β2-GP1 and leads to acceleration of aortic atherosclerosis (George *et al.* 1998a; Afek *et al.* 1999). Furthermore, transfer of lymph node cells reactive for human β2-GP1 to LDL-receptor-deficient mice promoted fatty streak formation (George *et al.* 2000). These findings suggest that an immune response against β2-GP1 may promote atherogenesis. Indeed, antibodies against β2-GP1 (Ranzolin *et al.* 2004; Romero *et al.* 1998) as well as antiphospholipids antibodies in general (Bili *et al.* 2000; Vaarala *et al.* 1995) have been shown to correlate positively with cardiovascular disease and its complications.

2.1.3 Heat shock proteins

Heat shock proteins (HSPs) act as chaperones and are involved in protein folding, intracellular transport, and controlled breakdown of proteins, and they are produced in large amounts by injured cells (reviewed by Xu 2002). HSPs are highly conserved in evolution, and

accordingly, human HSP60 can cross-react immunologically with mycobacterial HSP65 and chlamydial HSP60. Thus, protective humoral and cellular immune responses toward micro-organisms may be misdirected against human HSPs expressed by the stressed cells of the arterial wall (Perschinka *et al.* 2003; Mayr *et al.* 1999). Indeed, various risk factors of atherosclerosis, such as hemodynamic stress and oxLDL can induce HSP60 and also HSP70 expression in endothelial cells (Hochleitner *et al.* 2000; Zhu *et al.* 1994), macrophages (Frostegård *et al.* 1996), and smooth muscle cells (Xu *et al.* 2000; Zhu *et al.* 1995). Accordingly, HSP60 and also HSP70 are highly expressed in various cell types in atherosclerotic lesions of humans and animal models of atherosclerosis, and their expression correlates with the severity of atherosclerosis. To support the role of HSPs as an antigenic structures in atherogenesis, a number of studies have shown that antibodies against either human or microbial HSPs are associated and predict cardiovascular disease and its complications (reviewed by Xu 2002). In addition, human carotid atherosclerotic plaques have been shown to contain a population of T cells that specifically respond to chlamydial HSP60 (Mosorin *et al.* 2000; Curry *et al.* 2000), and recently a population of CD4+ T cells reacting with human HSP60 was also demonstrated in such lesions (Benagiano *et al.* 2005). Moreover, parenteral immunization with HSP60/65 has been shown to aggravate atherosclerosis in rabbit and mouse models of atherosclerosis (Afek *et al.* 2000; George *et al.* 1999b; Xu *et al.* 1992) and to lead to vascular inflammation and infiltrates of HSP60 reactive T cells (Xu *et al.* 1993). In addition to activating an adaptive immune response, HSPs have also been shown to directly activate the innate immune response via Toll-like receptors (TLR) (Ohashi *et al.* 2000).

2.1.4 Infectious agents

The role of infectious agents as antigens in atherogenesis is supported by the presence of various infectious agents in the arterial wall and by the seroepidemiological studies reporting an association between pathogen-specific antibodies and atherosclerosis. In this regard, most interest has focused on the role of *Chlamydia pneumoniae*, Herpes viruses, *Helicobacter pylori*, and oral pathogens. Yet, it is not clear whether the pathogens in the arterial wall are causal agents or only "innocent bystanders", and it has also been suggested that chronic low-grade infection or recurrent infections, even at extravascular sites of the body, may increase the risk for atherosclerosis (Kiechl *et al.* 2001). Furthermore, it is also possible that the risk of atherosclerosis is not increased by a single infectious agent, but rather the total number of pathogens to which a person is exposed during his or her lifetime, i.e, the "bacterial burden" (Zhu *et al.* 2000). In line with this hypothesis, a recent study demonstrated that the atherosclerotic lesions of patients with coronary heart disease contained highly diverse bacterial DNA (Ott *et al.* 2006). However, lipid-driven animal models have established that infectious agents are not necessary for lesion development, but this does not rule out the possibility, that infectious agents or an immune response toward them could still have a modifying function in atherosclerosis (Wright *et al.* 2000).

Of the above-mentioned pathogens, *Chlamydia pneumoniae* is the most extensively studied. It is an intracellular pathogen, which is suggested to be transported to atherosclerotic lesions by circulating monocytes from the respiratory tract. Indeed, *C. pneumoniae* has been detected in atherosclerotic plaques by various techniques, including immunohistochemistry, PCR, electron microscopy, and *in situ* hybridization, and it has also been successfully cultured from these lesions, while it has been very rarely demonstrated in healthy arterial wall (reviewed by Boman & Hammerschlag 2002). Furthermore, human atherosclerotic lesions have been shown to contain T cells that recognize *C. pneumoniae* (Mosorin *et al.* 2000; Curry *et al.*

2000), suggesting that this pathogen may activate the immune response locally in plaque. Antibodies against *C. pneumoniae* are prevalent in the population, and the results regarding their role and possible predictive value in atherosclerosis have been controversial. Although a vast number of cross-sectional studies initially demonstrated a strong correlation between elevated titers of anti-chlamydial antibodies and cardiovascular diseases, more recent prospective studies as well as their meta-analysis failed to detect such association (Bloemenkamp *et al.* 2003; Danesh *et al.* 2000), suggesting that a strong causal relationship is unlikely. This is in accordance with the recent trials examining the effect of anti-chlamydial antibiotic treatment on the clinical endpoints of cardiovascular diseases, which failed to demonstrate a beneficial role for this therapy in secondary prevention (Andraws *et al.* 2005). However, these negative results do not fully exclude a role of *C. pneumoniae* infection in atherogenesis, since *C. pneumoniae* may remain in a dormant, intracellular phase for long periods, during which it is resistant to eradication by the immune system as well as by antibiotic therapy. Indeed, various studies on experimental animals have shown that *C. pneumoniae* infection, superimposed on hypercholesterolemia, accelerates the development of atherosclerosis (Moazed *et al.* 1999; Laitinen *et al.* 1997; Muhlestein *et al.* 1998; Pislaru *et al.* 2003), although contradictory results have also been reported (Aalto-Setälä *et al.* 2001; Caligiuri *et al.* 2001). In addition, a very recent study showed that, during acute coronary syndrome, levels of both chlamydial lipopolysaccharide and CRP were increased and showed significant correlation (Tiirola *et al.* 2006). Their decrease after coronary event suggested that chlamydial lipopolysaccharide was released from arterial wall during event and participated in the induction of CRP production.

The interest in oral pathogens in the pathogenesis of atherosclerosis was inspired by the finding that patients with periodontal disease have a higher incidence of complications of atherosclerosis (Scannapieco *et al.* 2003). Similarly, elevated antibody levels against a periodontal pathogen, *Porphyromonas gingivalis*, have correlated with cardiovascular disease (Pussinen & Mattila 2004). Although it has been suggested that oral infections may only reflect confounding common risk factors of atherosclerosis or provide a focus of chronic inflammation in the body, recent evidence indicates that various periodontal pathogens are present in atherosclerotic lesions (Haraszthy *et al.* 2000) and can locally activate an immune response, as demonstrated by the presence of *P. gingivalis*-reactive T cells in plaques (Choi *et al.* 2002). Finally, *P. gingivalis* has been shown to accelerate atherosclerosis in animal models (Lalla *et al.* 2003; Jain *et al.* 2003; Li *et al.* 2002).

In addition to *Chlamydia pneumoniae* and periodontal pathogens, *Helicobacter pylori* and various viruses belonging to the herpesvirus family (HSV-1,-2, EBV, and CMV) have been detected in human lesions (Kaplan *et al.* 2005; Raza-Ahmad *et al.* 1995; Ibrahim *et al.* 2005). Furthermore, some reports exist on the correlation between seropositivity against EBV or *Helicobacter pylori* and cardiovascular diseases (Mendall *et al.* 1994; Musiani *et al.* 1990) and on the correlation between seropositivity for CMV and progression of atherosclerosis, especially in transplanted hearts or after coronary atherectomy (Danesh *et al.* 1997). Atherosclerotic lesions have also been shown to contain a T cell population that responds to EBV in a MHC class II-restricted manner (Boer de *et al.* 2005), and cytomegalovirus infection has been shown to increase atherosclerosis in apoE$^{-/-}$ mice (Hsich *et al.* 2001).

2.2 Infiltration of inflammatory cells into arterial intima

Infiltration of leukocytes, notably monocytes and lymphocytes, into the developing lesions is one of the initial events in atherogenesis. The process of leukocyte recruitment into the arterial wall is a multi-step process, which is dependent on the interaction between leukocytes and endothelium as well as the expression of various chemokines. Infiltration of inflammatory cells into the intima seems to be important for atherogenesis, since disruption of this process by genetic deletion of endothelial adhesion molecules, leukocyte integrins, or chemotactic molecules and their receptors leads to decreased atherosclerosis in animal models (see Table 4).

Experimental evidence shows that, very soon after the introduction of an atherogenic diet, leukocytes are attached to the endothelium of rabbit vessel wall (Poole & Florey 1958). In this initial step, leukocytes bind to endothelial P- and E-selectins, resulting in tethering and rolling of the cells along the vessel wall. Next, leukocytes adhere more firmly to the endothelium. This step is dependent on the activation of leukocytes by chemoattractants released from the tissues followed by binding of leukocyte integrins i.e. very late antigen-4 (VLA-4) and lymphocyte function-associated antigen-1 (LFA-1) to their endothelial counterparts, i.e. vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), respectively. Regarding atherosclerosis, endothelial cells have been shown to express VCAM-1 and ICAM-1 selectively in areas prone to lesion formation (Cybulsky & Gimbrone 1991; Nakashima *et al.* 1998). In addition, both selectins and VCAM-1 and ICAM-1 are strongly expressed in endothelium overlying developing plaques, whereas expression is much lower or non-existent in endothelium overlying healthy intima (Davies *et al.* 1993; Nakashima *et al.* 1998). Interestingly, the expression of these adhesion molecules by endothelial cells has been shown to increase in response to hemodynamic stress and also in response to plaque material, notably components formed during lipoprotein oxidation (Vora *et al.* 1997; Walpola *et al.* 1995; Frostegård *et al.* 1991; Watson *et al.* 1997). Recently, these early events of leukocyte-endothelium interactions have also been investigated *in vivo.* By using the modern technique of intravital microscopy, Eriksson *et al.* demonstrated that, in a mouse model, the rolling of leukocytes occurred on the periphery of atherosclerotic lesions but not in normal vessel wall, and that these leukocyte-endothelial interactions were transient and critically dependent on the expression of endothelial selectins (Eriksson *et al.* 2001). In addition to this integrin-dependent pathway, an integrin-independent pathway for leukocyte adhesion has also been described. This pathway involves the interaction between the membrane-bound chemokine fractalkine (CX3CL1) on endothelium and chemokine receptors CX3CR1 on monocytes, T lymphocytes, NK cells, and dendritic cells (Fong *et al.* 1998). Both fractalkine and its receptor have been shown to be expressed in atherosclerotic lesions (Wong *et al.* 2002). To support the importance of endothelial-leukocyte interactions in atherosclerosis, various studies have shown that genetic deletion of E-selectin, P-selectin, ICAM-1, VCAM-1, or CX3CR1 leads to decreased lesion size in mouse models of atherosclerosis (Lesnik *et al.* 2003; Combadiere *et al.* 2003; Collins *et al.* 2000; Dong *et al.* 1998).

After the adherence of leukocytes to the endothelium, different chemotactic molecules are required for the transmigration of attached leukocytes through the endothelial lining to the arterial wall. Chemotactic molecules include complement components (C3a and C5a; see section *Complement effectors*), chemotactic cytokines (chemokines), and modified lipoproteins. Chemokines constitute a family of structurally related chemotactic cytokines, which are classified into subgroups (CC, CXC, C, CXXXC) based on the position of N-

terminal cysteines, and they act through chemokine receptors on the target cells. A wide repertoire of both chemokines (e.g. MCP-1, RANTES, MIP-1α, MIP-1β, GRO-α, IL-8, IP-10, fractalkine, eotaxin) and their receptors (e.g. CCR2, CCR3, CXCR2, CX3CR1) have been shown to be expressed in atherosclerotic lesions (reviewed by Weber *et al.* 2004; Sheikine & Hansson 2004), and it has also been shown that platelets interacting with endothelium may release chemokines and subsequently increase the recruitment of leukocytes to atherosclerotic plaque (Hundelshausen von *et al.* 2001). Interestingly, upregulation of chemokine expression is observed in response to various stimuli associated with atherosclerosis such as minimally modified LDL (Miller *et al.* 2005), complement activation products (Torzewski *et al.* 1996), and alterations in shear stress (Shyy *et al.* 1994). In addition, oxLDL can also directly induce chemotaxis of human monocytes (Quinn *et al.* 1987). The current assumption suggests that the recruitment of leukocytes requires cooperation of multiple chemotactic factors in distinct steps, but understanding of the recruitment pathways for individual leukocyte subclasses is limited. It is known, however, that the selective recruitment of mononuclear cells to the arterial wall during early atherogenesis results from the expression of VLA-4 in monocytes and T lymphocytes, but not in granulocytes. Similarly to adhesion molecules, genetic deletion or pharmacologic blockade of various chemokines or their receptors results in decreased atherosclerosis in mouse models (see Table 4), thus emphasizing the importance of these molecules in atherogenesis.

2.3 Innate immunity in atherosclerosis

The innate, non-adaptive immune system, which contains both cellular and soluble components, provides a fast (from minutes to hours) but blunt response, and serves as "first-line" defense against infectious agents. Cells of the innate immune system are able to respond to pathogens via receptors called pattern recognition receptors (PRRs), which are able to recognize a restricted pattern of ligands called pathogen-associated molecular patterns (PAMP). These receptors are mainly expressed on cells of phagocytic origin, i.e. dendritic cells and macrophages. In addition to these receptors, the innate immune system comprises soluble factors such as the complement system, acute phase proteins, and cytokines, and also germ line-encoded antibodies, called natural antibodies (Cellular and Molecular Immunology, 5th Edition, 2005 by Abbas *et al.*). All these may act together in the arterial intima to eliminate locally accumulated harmful agents, but by performing their physiologically meaningful functions, the activated innate immune system may also contribute to the pathogenesis of atherosclerosis (Fig. 2).

2.3.1 Cells of innate immunity

Various types of innate immune cells (macrophages, dendritic cells, NK cells, mast cells, neutrophils, B1 cells) have been suggested to play a role in atherogenesis. These cells exert their effects by cell-mediated cytotoxicity or by release of various cytokines, enzymes or antibodies, all of which are likely to have implications for the pathogenesis of atherosclerosis. In addition, dendritic cells and macrophages bridge innate and adaptive immunity by presenting antigens on their surface MHC class II molecules for recognition by cells of the adaptive immune system (lymphocytes).

Macrophages

Macrophages are the most abundant type of inflammatory cells in atherosclerotic lesions (approximately 80% of leukocytes), and they are present at all stages of atherosclerosis.

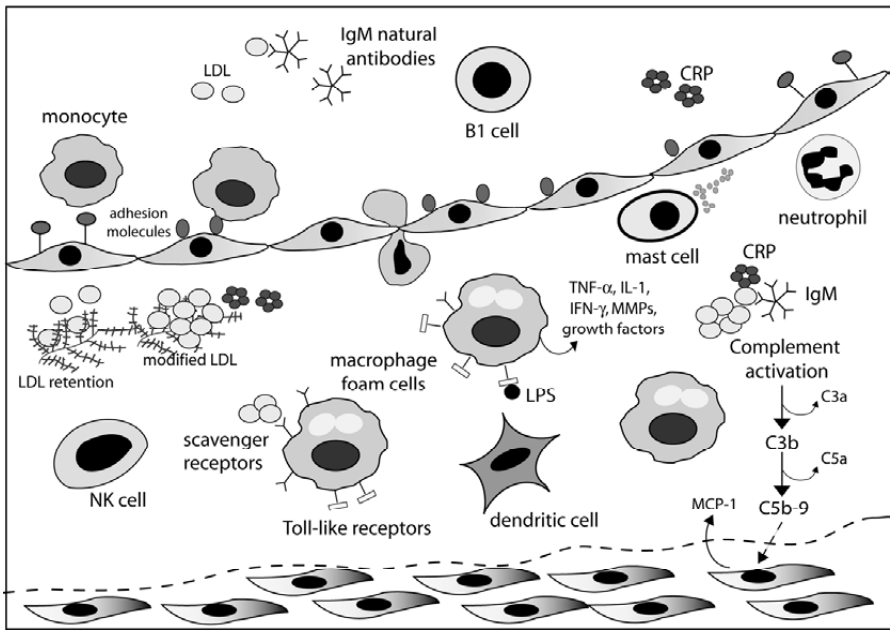

Fig. 2. Schematic representation of the players of the innate immune system in atherosclerotic lesions. Cells of innate immunity, notably monocytes, enter the arterial wall early in atherogenesis. In the arterial wall, monocyte-derived macrophages recognize foreign material by various pattern recognition receptors, which lead to their activation. In addition, other components of the innate immune system, e.g. acute phase reactant CRP, natural antibodies, and complement system, are present in the intima.

Macrophages derive from blood monocytes, which under the influence of growth factors (monocyte colony-stimulating factor; M-CSF or granulocyte-macrophage colony-stimulating factor; GM-CSF) differentiate into tissue macrophages. These, in turn, are transformed into foam cells upon uptake of modified lipoproteins. Macrophages are important players of inflammation and the innate immune response in atherosclerosis, and they may contribute to various stages of atherogenesis by promoting local inflammatory reactions. Thus, macrophages produce pro-inflammatory cytokines (i.e. IL-1β, IL-6, IL-8, TNF-α), oxygen radicals, growth factors, leukotrienes, complement factors (reviewed by Østerud & Bjørklid 2003), and enzymes capable of modifying lipoproteins (Hakala *et al.* 2003). In addition, macrophages may also promote plaque destabilization through production of proteolytic enzymes, e.g. matrix metalloproteinases (Galis *et al.* 1994). In addition, by serving as antigen-presenting cells, macrophages may contribute to the activation of adaptive immune responses. The essential role of macrophages in atherogenesis is supported by the finding that apoE$^{-/-}$ mice deficient for M-CSF, which lack tissue macrophages, develop significantly less atherosclerosis than control mice (Smith *et al.* 1995).

As players of the innate immune response, macrophages recognize foreign material by a variety of receptors that bind to target structures either indirectly via opsonic molecules or via direct interactions. The former process is based on the recognition of opsonic antibodies or complement products by macrophage Fcγ and complement receptors, respectively. In contrast, the latter process is based on the recognition of foreign structures by a repertoire of PRRs, notably scavenger receptors and Toll-like receptors (TLR). At least two of the scavenger receptors, scavenger receptor A (SR-A) and CD36, are of particular importance for the innate immune response in plaque. Thus, both receptors are involved in the uptake of modified lipoproteins and the transformation of macrophages into foam cells (reviewed by

Greaves & Gordon 2005). In addition, the uptake process via SR-A does route the antigen for presentation on MHC class II molecules and for further recognition by cells of the adaptive immune system (Nicoletti *et al.* 1999). In addition to modified LDL, scavenger receptors can recognize e.g. apoptotic cells and in pathogenic organisms (reviewed by Peiser *et al.* 2002). The role of Toll-like receptors, especially TLR4, has gained much attention in atherogenesis during recent years (reviewed by Tobias & Curtiss 2005). TLRs are a family of pattern recognition receptors with at least 10 members identified in mammals so far. The entire spectrum of these receptors has been detected in endothelial cells of the normal arterial wall, and at least the expression of TLR1, TLR2, and TLR4 is significantly increased in atherosclerotic plaques, in which they are mainly expressed by monocyte-macrophages and dendritic cells, but also by endothelial cells (Edfeldt *et al.* 2002; Xu *et al.* 2001). Interestingly, atherosclerotic lesions also contain various ligands that have been suggested to signal via TLRs, i.e. minimally modified LDL (Miller *et al.* 2003b), *C. pneumoniae* (Prebeck *et al.* 2001), periodontal pathogens (Hajishengallis *et al.* 2002), and human HSP60 (Ohashi *et al.* 2000). Ligation of TLRs leads to the activation of NF-κB- or IRF-3-responsive genes with subsequent synthesis of various pro-inflammatory cytokines, chemokines, adhesion molecules as well as co-stimulatory molecules (reviewed by Michelsen *et al.* 2004a), which can promote inflammation and activation of adaptive immune response in atherosclerotic lesions. Regarding the role of pattern recognition receptors in atherosclerosis, various animal studies have shown that genetic deletion of either scavenger receptors or TLR4 significantly decreases the extent of atherosclerosis (Suzuki *et al.* 1997; Febbraio *et al.* 2000; Björkbacka *et al.* 2004; Michelsen *et al.* 2004b). Furthermore, the TLR Asp299Gly polymorphism that attenuates receptor signaling is associated with decreased atherosclerosis and a reduced risk of acute coronary events in humans (Kiechl *et al.* 2002; Ameziane *et al.* 2003). However, some studies have failed to find such an association (Yang *et al.* 2003), and a recent report even suggested an increased risk of myocardial infarction in patients with Asp299Gly polymorphism (Edfeldt *et al.* 2004).

Dendritic cells

Dendritic cells are highly efficient antigen-presenting cells, which, in addition to expressing a high density of MHC class II molecules on their surface, also express a set of co-stimulatory molecules (e.g. B7-1 or B7-2) needed for the activation of naïve T cells. In addition to traditional T cells, dendritic cells can also present lipid antigens on CD1d for recognition by natural killer T cells (NKT cells)(Bobryshev & Lord 2002). A small number of dendritic cells have been shown to be present in the normal vessel wall in vascular-associated lymphoid tissue (VALT), especially in atherosclerosis-prone areas (Wick *et al.* 1997; Lord & Bobryshev 1999), and during the development of atherosclerotic lesion their number is further increased (Bobryshev & Lord 1995). In addition to entering the intima through luminal endothelium, it has also been suggested that dendritic cells may enter the intima via *vasa vasorum* (Bobryshev & Lord 1998) or differentiate from infiltrated monocytes (Randolph *et al.* 1998). It has also been shown that adhesion and endothelial transmigration of dendritic cells is increased by stimuli known to accelerate atherogenesis such as oxLDL (Weis *et al.* 2002). Accumulating evidence indicates that dendritic cells are involved in antigen processing and presentation *in vivo* in atherosclerotic tissue. Thus, in atherosclerotic plaques, dendritic cells were observed in contact with T cells, which phenomenon was accompanied by the expression of ICAM-1 and VCAM-1, both of which are involved in dendritic cell-mediated T cell activation (Bobryshev & Lord 1998). Furthermore, similar colocalization was observed in the shoulder areas of vulnerable plaques, and in contrast to initial lesions, these dendritic cells exhibited a mature phenotype, indicating their functional activity, and suggesting that antigen presentation to and activation of T cells may affect the stability of plaque (Yilmaz *et al.*

2004). In addition, in the shoulder region of atherosclerotic lesions, CD1d-expressing dendritic cells were in direct contact with NKT cells, suggesting that presentation of lipid antigens also occurs in plaques (Bobryshev & Lord 2005a).

Natural killer cells

Natural killer cells (NK cells) are bone marrow-derived lymphocytes, which can function either by releasing cytoplasmic granules containing perforin and granzymes, which mediate direct cell-mediated cytotoxicity, or by producing pro-inflammatory cytokines, most importantly IFN-γ. A small number (approximately 0.1-0.5 % of the total T cell population) of NK cells have already been detected in the vessel wall in the early phases of atherosclerosis, and their number does not seem to increase during the progression of atherosclerosis (Millonig *et al.* 2002; Bobryshev & Lord 2005b). In contrast, NK cells have been widely detected in arterial aneurysms and even suggested to play a role in their formation (Seko *et al.* 1997). Despite the small number of NK cells in atherosclerotic lesions, elevated levels of circulating NK cells have correlated with severe atherosclerosis (Clerc & Rouz 1997). Several studies on experimental animals that have tried to shed light on the role of NK cells in atherogenesis have yielded controversial results. In $LDLR^{-/-}$ mice, perforin deficiency and $Lyst^{beige}$ mutation, both of which result in defective NK cell-mediated cytotoxicity, had different effects on atherogenesis; the former had no effect, whereas the latter increased atherosclerosis (Schiller *et al.* 2002). In contrast, a recent study demonstrated that a lack of functional NK cells results in significant reduction of lesion size in $LDLR^{-/-}$ mice (Whitman *et al.* 2004). Hence, it is possible that NK cells are involved in the pathogenesis of atherosclerosis, but the effect does not seem to depend on the cytolytic effects of NK cells.

Mast cells

Mast cells are cells of innate immunity that are traditionally known for their actions in allergic reactions. Upon activation, mast cells degranulate, i.e. release cytoplasmic secretory granules containing vasoactive substances such as histamine, proteolytic enzymes such as tryptase and chymase, inflammatory cytokines, growth factors, and heparin proteoglycans (Maurer *et al.* 2003). In normal coronary intima, only a few mast cells are present in the subendothelial part. The number of mast cells increases during atherogenesis, and in advanced lesions they comprise 0.5-1% of the total intimal cell population (Kaartinen *et al.* 1994a). In advanced lesions, mast cells are mainly concentrated in the shoulder areas as part of the inflammatory infiltrate (Kaartinen *et al.* 1994a) and also occur around intimal neovessels (Kaartinen *et al.* 1996). Atherosclerotic intima contains various substances that are known to activate mast cells, e.g. immune complexes (Ylä-Herttuala *et al.* 1994) and complement-derived anaphylatoxins (Vlaicu *et al.* 1985a). Interestingly, a very high proportion of mast cells (85%) in the shoulder area of an advanced atherosclerotic lesion are degranulated, reflecting the activation of these cells. This finding together with studies showing that mast cell degranulation and activation lead to the release of enzymes and cytokines that may weaken the structure of plaque (reviewed by Lindstedt & Kovanen 2004) suggests that mast cells may have a role in acute coronary events. Indeed, Kaartinen *et al.* detected an increased number of mast cells in the culprit lesions of patients with unstable angina (Kaartinen *et al.* 1998), and infiltrates of activated mast cells have been detected at the site of atheromatous erosion or rupture in myocardial infarction (Kovanen *et al.* 1995). During atherogenesis, mast cells may also interfere with lipid metabolism and thus promote accumulation of lipids both intra- and extracellularly in lesions, regulate T cell and macrophage responses, and promote endothelial dysfunction (reviewed by Vanderlaan & Reardon 2005). In contrast, mast cells may also exert

anti-atherogenic functions such as inhibition of collagen-induced platelet aggregation (Lassila *et al.* 1997) and prevent oxidation of LDL (Lindstedt 1993). To better define the significance of mast cells in atherogenesis, if any, animal studies with mast-cell-deficient mice and with mice with excessive numbers of mast cells are required.

Neutrophils

Neutrophils are the cells of the innate immune system that are characteristic of acute inflammation. They are able to phagocytose and destroy target structures and release reactive oxygen species, cytokines, antimicrobial peptides such as defensins, and various enzymes, some of which (e.g. MMP-8 and elastase) possess proteolytic activity. Neutrophils are rarely detected in stable human atherosclerotic plaques, but they are prevalent in the culprit lesions of patients with unstable angina as well as in the eroded and ruptured plaques of patients with myocardial infarction (Naruko *et al.* 2002). However, it is noteworthy, that since the life span of neutrophils in tissues is short (from a few hours to few days), even a small number of detected neutrophils in histological sections may reflect a more significant role for these cells in plaque *in vivo*. This is supported by a finding, which showed that leukocytes interacting with atherosclerotic endothelium are predominantly neutrophils (Eriksson *et al.* 2001). In addition, recent study by Sugano and coworkers showed that activated polymorphonuclear leukocytes from hypercholesterolemic patients produced increased amounts of superoxide, impaired endothelial function and showed increased adhesiveness to cultured endothelial cells (Sugano *et al.* 2005). Of interest, it has also been shown that upon intraluminal release of myeloperoxidase from neutrophils, this enzyme binds to endothelial cells and undergoes transcytosis to the subendothelial matrix (Baldus *et al.* 2001), where it can be involved in the oxidation of lipoproteins, for instance. Interestingly, it has been shown that neutrophils become activated as they traverse the coronary vascular bed in patients with unstable angina (Buffon *et al.* 2002). Moreover, a high number of circulating neutrophils has been reported to be an independent predictor of coronary heart disease and myocardial infarction (Madjid *et al.* 2004).

B1 cells and natural antibodies

Natural antibodies are defined as antibodies that circulate in healthy individuals even in the absence of exogenous antigenic stimulation. They are produced by so-called B1 lymphocytes, are predominantly of IgM type, and contain highly-conserved sequences that recognize a variety of exogenous antigens as well as altered self structures. Thus, in addition to providing protection against invading pathogens, natural antibodies have been considered to have a "housekeeping" function in the removal of senescent and apoptotic cells and other self-antigens, and thus to protect from autoimmunity (reviewed by Baumgarth *et al.* 2005). Indeed, the role of these antibodies might be emphasized in conditions that involve increased generation of non-self structures, as during atherogenesis.

Natural antibodies were first linked to atherosclerosis when a panel of monoclonal IgM antibodies binding to oxLDL, more specifically the phosphoholine (PC) head group of oxidized phospholipids, were cloned from spleens of $ApoE^{-/-}$ mice (Palinski *et al.* 1996; Hörkkö *et al.* 1999). These antibodies (EO6 as a prototype) were able to stain both rabbit and human atherosclerotic tissue and recognize epitopes in human plasma LDL. *In vitro* studies showed that they also inhibited the uptake of oxLDL and apoptotic cells by macrophage scavenger receptors (Hörkkö *et al.* 1999; Chang *et al.* 1999). Further characterization of these IgM antibodies revealed that they were totally homologous (V_H/V_L-region) with the T15 natural antibodies (Shaw *et al.* 2000) that are known to bind to phosphocholine molecules

present in the pneumococcal cell wall and confer to mice optimal protection against *Streptococcus pneumoniae* infection. Moreover, E06 and T15 were cross-reactive, since both bound to oxLDL as well as to the pneumococcal cell wall polysaccharide. The membranes of apoptotic cells were also shown to contain enriched amounts of PC-containing oxidized phospholipids that were recognized by natural antibodies (Chang *et al.* 2004). These findings suggested that IgM autoantibodies against oxidation-specific epitopes were actually natural antibodies present at birth and belonged to the innate immune response. Furthermore, these studies suggested that oxidation-specific neoepitopes represent PAMPs that are recognized by the components of the innate immune system, i.e. natural antibodies, scavenger receptors, and CRP.

Natural antibodies have been suggested to play a protective role in atherogenesis. Thus, immunization of $LDLR^{-/-}$ mice with *S. pneumoniae*, which led to a significant increase in the plasma levels of IgM recognizing both oxLDL and pneumococcal cell wall polysaccharide and to expansion of T15 IgM-secreting B cells in the spleen, protected mice from atherosclerosis (Binder *et al.* 2003). In addition, immunization of $LDLR^{-/-}$ mice with MDA-modified LDL led to reduced atherosclerosis, and this effect was associated with the Th2 type adaptive immune response, which also boosted the production of natural T15/E06 antibodies via production of IL-5. In addition, IL-5 deficiency was found to increase atherosclerosis in this mouse model, suggesting that the anti-atherogenic effects of immune activation against oxLDL could be partly mediated via a cytokine-induced increase in the production of T15/E06 natural antibodies (Binder *et al.* 2004).

2.3.2 Acute-phase proteins

Acute-phase reactants are plasma proteins, the level of which increase or decrease in response to infection, trauma or other inflammatory conditions, and whose concentration may also reflect the intensity of the process (Table 1). Acute-phase proteins are mainly produced by hepatocytes, but extrahepatic synthesis may also occur. As players of the innate immune response, acute-phase proteins may, for instance, enhance the activation of complement, promote the production of pro-inflammatory cytokines, assist in phagocytosis, and also increase the infiltration of leukocytes to the site of inflammation (reviewed by Gabay & Kushner 1999).

In recent years, demonstration of a direct association between mildly elevated serum levels of various acute-phase proteins such as C-reactive protein (CRP), serum amyloid A, and fibrinogen and the risk of cardiovascular disease have been widely reported (reviewed by Ridker *et al.* 2004). It has been speculated that these mildly elevated levels of acute-phase proteins may reflect the presence of low-grade inflammation in the arterial tree or chronic infection associated with cardiovascular risk, or even that these proteins may be directly involved in the pathogenesis of atherosclerosis and its complications. Regardless of the underlying cause, they may provide the clinician with additional diagnostic and prognostic tools.

The role and prognostic value of C-reactive protein in cardiovascular diseases has been under intensive research during recent years. CRP is an acute-phase protein, in which five identical subunits form a pentameric structure, and being pattern recognition receptor, it typically binds to the structures found on the surface of pathogens such as phosphocholine (reviewed by Black *et al.* 2004). Highly sensitive assays enable accurate determination of even very low plasma levels of CRP, and various prospective studies have shown that hsCRP is an

Table 1. Acute-phase proteins whose plasma concentrations increase during inflammatory process (Gabay & Kushner 1999)

Complement system	**Transport proteins**
C3	Ceruloplasmin
C4	Haptoglobin
C9	Hemopexin
Factor B	**Participants in inflammatory responses**
C1 inhibitor	Secretory phospholipase A_2
C4b-binding protein	Lipopolysaccharide-binding protein
mannose-binding lectin	Interleukin-1-receptor antagonist
Vitronectin	Granulocyte colony-stimulating factor
Coagulation and fibrinolytic system	**Others**
Fibrinogen	C-reactive protein
Plasminogen	Serum amyloid A
Tissue plasminogen activator	α1-Acid glycoprotein
Urokinase	Fibronectin
Protein S	Ferritin
Plasminogen-activator inhibitor 1	Angiotensinogen
Antiproteases	
α1-Protease inhibitor	
α1-Antichymotrypsin	
Pancreatic secretory trypsin inhibitor	
Inter-α-trypsin inhibitor	

independent risk factor of cardiovascular disease and myocardial infarction even in apparently healthy individuals (reviewed by Torres & Ridker 2003). Based on population samples, hsCRP levels of less than 1 mg/ml, 1-3 mg/ml, and more than 3 mg/ml should be interpreted as indicative of low, moderate, and high risk for cardiovascular disease, respectively. However, the usefulness of CRP as a novel additional marker of cardiovascular risk is still under debate, since a recent meta-analysis of previously published studies contradicted this view by showing that CRP was only a modest predictor of coronary events and added only marginally to the predictive value of the established risk factors (Danesh *et al.* 2004). However, the Centers for Disease Control and Prevention and the American Heart Association recently recommended that patients with an intermediate risk for developing CVD could benefit from the measurement of plasma CRP levels (Pearson *et al.* 2003).

Whether CRP is merely a marker of inflammation or whether it could have an active role in atherogenesis is not known. However, the presence and expression of CRP in atherosclerotic plaques and the reported *in vitro* pro- and anti-inflammatory effects have suggested that CRP may affect atherogenesis. Indeed, the expression of CRP is increased in atherosclerotic plaques compared to healthy vessels (Yasojima *et al.* 2001b), and the plaques of patients with unstable angina showed significantly higher immunoreactivity to CRP than the plaques of patients with stable angina (Ishikawa *et al.* 2003). It has also been suggested that this local production of CRP could account for the elevated plasma levels, since the level of CRP was significantly higher in the cardiac vein than in the coronary arteries (Ishikawa *et al.* 2004). In addition, the translesional CRP gradient was greater in unstable than stable angina, suggesting that increased production of CRP by the lesional cells might reflect increased inflammation and instability of plaque (Inoue *et al.* 2005). Regarding the possible effects of CRP on plaque, both anti- and pro-atherogenic effects have been reported. Thus, CRP can be involved in the

clearance of modified self-structures by increasing their uptake either directly via Fcγ receptors or by activating the classical complement pathway with ensuing uptake of target structures via complement-derived opsonins (C1q and iC3b). Of the structures present in lesions, CRP has been shown to bind and increase the uptake of apoptotic cells and oxidized or enzymatically modified LDL (Chang *et al.* 2002; Gershov *et al.* 2000; Bhakdi *et al.* 1999; Taskinen *et al.* 2002). Furthermore, CRP has been shown to modulate the activation of the complement by enzymatically modified LDL in such a way that the detrimental end product C5b-9 is not generated (Bhakdi *et al.* 2004). In contrast, CRP also induces the expression of adhesion molecules, chemokines, and endothelin-1 by endothelial cells, is chemotactic for monocytes, and induces SMC apoptosis (reviewed by Paul *et al.* 2005). However, it is noteworthy that at least cell culture studies on the effects of CRP on intimal cell types should be interpreted with caution, since the results may be affected by endotoxin or azide contamination of commercial CRP preparations (Taylor *et al.* 2005). Recent studies with experimental animals have provided ambiguos findings about the role of CRP in atherogenesis. Thus, the study by Paul *et al.* showed that transgenic expression of human CRP accelerates the development of atherosclerosis in male but not female apoE$^{-/-}$ mice (Paul *et al.* 2004), whereas three other studies failed to reveal any such effect for either human or rabbit CRP transgene in apoE$^{-/-}$ (Hirschfield *et al.* 2005; Reifenberg *et al.* 2005) and apoliporotein E*3 Leiden (Trion *et al.* 2005) mice. These conflicting results may be explained by the fact that, in the study of Paul *et al.*, the CRP concentrations were extraordinarily high compared to the other studies using the same transgenic strain, possibly reflecting a concurrent inflammatory process. In addition, it seemed that, in apoE$^{-/-}$ mice, CRP was not able to activate the complement system, which is one of its primary functions, and these results should hence be interpreted and extrapolated to humans with caution (Reifenberg *et al.* 2005).

2.3.3 Complement system

Introduction

The father of the complement is Dr. Jules Bordet, who in the late 19th century described a heat-labile activity in serum that could complement the ability of a specific antibody to cause lysis of bacteria. Today this heat-labile activity is known to comprise dozens of serum proteins and is known as the complement system. Traditionally, complement is regarded to be part of the innate immunity, although it has recently also become regarded as a key modulator of adaptive immunity. Complement is known to function both in the defence against microbes and in the clean-up of tissues, e.g. in the disposal of immune complexes and apoptotic cells. To exert its biological activities, the complement system needs to be activated, after which it generates a variety of functional molecules, i.e. opsonins, anaphylatoxins, and terminal membrane attack complex, all of which act separately or together to destroy the target structure (Cellular and Molecular Immunology, 5th Edition, 2005 by Abbas *et al.*) In addition, as a bridge between innate and adaptive immunity, the complement system modulates both B and T cell responses to antigens through the complement receptors and regulators present on the lymphocyte surface (reviewed by Morgan *et al.* 2005).

The complement system consists of approximately 30 proteins, including complement proteins and fluid-phase and membrane-bound regulators. A number of complement proteins are proteolytic enzymes that also in themselves become activated by proteolytic cleavage. Thus, a component of the system often serves as a substrate of a previous component and then, once activated, acts as an enzyme to activate the subsequent component. This pattern of sequential activation produces an expanding cascade of activity, which means that the activation of a single molecule will lead to thousands of molecules being generated in the

following steps. Thus, many regulatory mechanisms are needed to prevent uncontrolled complement activation and tissue damage.

The intitial finding of complement activation products in atherosclerotic lesions suggested that this mechanism of innate immunity is functional during atherogenesis. Since then, various structures capable of activation have been demonstrated in atherosclerotic lesions, and the effects of complement activation products on lesional cells have been widely investigated *in vitro*. Furthermore, animal studies have supported the role of the complement system in atherogenesis.

Complement activation

Depending on the nature of the activator, three different activation pathways, namely the classical, alternative, and lectin pathways, have been described. Each pathway utilizes different proteins to recognize the activators and to initiate the cascade. However, all three pathways converge at the level of C3 and share a common terminal pathway. In general, the components of the classical pathway and the terminal pathway are designated with the letter C followed by a number such as C4, whereas the components of the alternative pathway are called "factors" and are identified with a single letter such as factor D. The split products of the components that are generated by proteolytic cleavage are distinguished from their precursors by suffix letters such as C4b. The complement activation is reviewed in Cellular and Molecular Immunology, 5th Edition, 2005 by Abbas *et al.* A schematic view of complement activation and its regulators is presented in Fig. 3.

The classical complement pathway is primarily activated by antigen-bound IgG or IgM molecules, but CRP (Siegel *et al.* 1974), nucleic acids (Jiang *et al.* 1992), or apoptotic cells (Nauta *et al.* 2002b) can also initiate its activation. The activation involves recognition and binding of C1q to the activators with subsequent proteolytic activation of C1q-associated C1r and C1s. C1s is a serine protease that cleaves C4 with ensuing release of small peptide C4a and generation of larger C4b, which is capable of binding to nearby surfaces containing hydroxyl or amino groups. Surface-bound C4b binds C2, which can also become cleaved by C1s. While the generated C2a is released, C2b remains bound to C4b, thus forming the classical pathway C3 convertase C4b2b. This convertase, in turn, is cabable of cleaving C3 to C3a and C3b, and when C3b binds to the C4b2b complex, a functional C5 convertase is formed.

The alternative complement pathway is continuously undergoing low-grade spontaneous activation to generate C3b i.e. "C3 tickover". The generated C3b has the potential to bind to amino and hydroxyl groups on the nearby surfaces. If C3b binds to nonactivating surfaces, i.e. host cells, is rapidly inactivated by factor H and factor I, whereas C3b bound to activating surfaces that do not express or lack complement inhibitors, i.e. pathogens or other foreign surfaces, persists. The membrane-fixed C3b next binds factor B, which then becomes cleaved by factor D to Bb. The C3bBb complex is alternative pathway C3 convertase, which is stabilized by properdin (C3bBbP), and which is able to to cleave even more C3 molecules. This C3b-Bb-factor D circle is called "amplification loop", and it is necessary for efficient complement activation. The amplification loop also attends the augmentation of the complement activation initiated by the classical pathway (Müller-Eberhard 1988). An alternative pathway C5 convertase is formed when C3b binds to C3bBb (C3b2Bb).

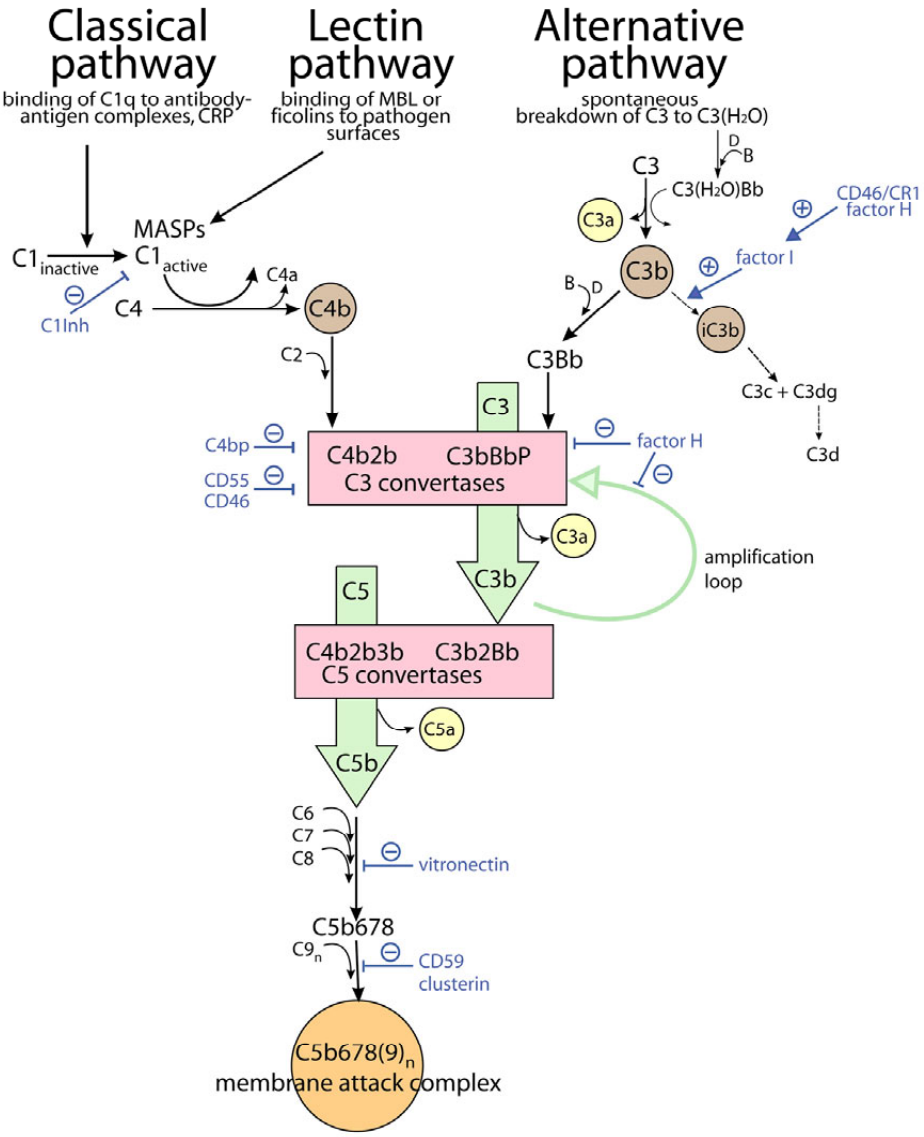

Figure 3. Schematic representation of the complement system and its regulators. Opsonins generated during complement activation are colored brown and anaphylatoxins yellow. Complement regulators are colored blue. Adapted from Oksjoki *et al.* (2003).

Finally, the lectin pathway of activation involves the binding of mannose-binding lectin (MBL) or ficolins, which are typical pattern recognition receptors, to carbohydrate groups on the surface of bacterial cells. This recognition is followed by the activation of associated enzymes, MBL-associated serine proteases (MASPs), which can cleave C4 and C2 with subsequent activation of the classical complement pathway (reviewed by Fujita *et al.* 2004).

Regardless of the initial pathway of activation, all pathways converge with the activation of the central C3 component. If the complement cascade is not regulated at this level, it proceeds to the activation of the terminal common pathway. In this pathway, C5 is cleaved into C5a and C5b by either the classical or alternative pathway C5 convertases, C4b2b3b and C3b2Bb, respectively. The ensuing sequential binding of C6, C7 and C8 to the emerging C5b678 complex does not require proteolytic cleavage, and the resulting complex guides the polymerization of as many as 10-16 molecules of C9 into a tube inserted into the lipid bilayer of the plasma membrane. The resulting pore-forming structure (C5b-9) is called “membrane-

attack complex", and it allows the passage of ions and small molecules into the cell with ensuing cell lysis

Since complement activation proceeds as an expanding cascade of activity, this easily explosive potential needs to be kept under strict control to avoid harmful tissue destruction. The regulatory mechanisms of the complement system are presented in the section *Complement regulators in atherosclerotic lesions.*

Complement activation products in atherosclerotic lesions

There is powerful evidence to indicate that the complement system is activated in human atherosclerotic, but not non-atherosclerotic, arterial wall. As early as 1979, Hollander and coworkers isolated complement C3 from the arterial wall and observed much higher C3 levels in atherosclerotic lesions than in normal vessel wall (Hollander *et al.* 1979). Subsequently, inactivation products of C3, more specifically C3c and C3dg, were also found in extracts of atherosclerotic lesions, suggesting that C3 had undergone proteolytic cleavage and activation (Seifert *et al.* 1991). Similarly, immunohistochemical stainings have demonstrated deposits of C3c as thin streaks and homogenous bands in the superficial intima (Vlaicu *et al.* 1985a), and in another study, C3 was also found in endothelial cells at sites of injury and in subendothelial intima (Hansson *et al.* 1984). Quantative determinations of complement components in atherosclerotic lesions have revealed that the complement proteins C1q, C3, C3a, C3c, C4, and C9 presented higher intima/serum ratios than the other serum proteins studied (albumin, transferrin, α1-antitrypsin, α2-macroglobulin, fibrinogen), suggesting preferential retention of these complement proteins in the intima (Vlaicu *et al.* 1985c). Furthermore, the observed increase in the intima/serum ratio may also partly result from increased local production of complement proteins in atherosclerotic lesions (Yasojima *et al.* 2001a; Yasojima *et al.* 2001b).

In addition to individual complement components, the terminal membrane-attack complex (MAC, C5b-9) has also been detected in atherosclerotic lesions. Similarly to complement components, C5b-9 is not present in normal arterial wall. However, as shown in cholesterol-fed rabbits, deposition of C5b-9 in the intima is an early event in atherogenesis, since it correlates temporally with cholesterol accumulation in the subendothelium and occurs before monocyte infiltration and foam cell formation (Seifert *et al.* 1989). Indeed, C5b-9 has been demonstrated in atherosclerotic lesions at all stages (Torzewski *et al.* 1997; Vlaicu *et al.* 1985a), and analysis of lesional extracts revealed that the amount of C5b-9 correlates with lesion severity (Niculescu *et al.* 1987a). In addition, the majority of lesional C5b-9 is in a membraneous, cytolytic form, suggesting that it has been formed *in situ* in the lesions (Seifert *et al.* 1990). Immunohistochemical analyses have shown that, throughout lesion development, C5b-9 appears as granular deposits in the deep parts of the intima adjacent to the media and occasionally also in the inner third of the media (Torzewski *et al.* 1997; Niculescu *et al.* 1987c). In the deep area, C5b-9 has been shown to colocalize with smooth muscle cell α-actin (Torzewski *et al.* 1997), CRP (Torzewski *et al.* 1998a), apoptotic cells (Niculescu *et al.* 2004) as well as modified lipids (Torzewski *et al.* 1998b). In line with these results, smooth muscle cells have been suggested to be the initial target of the complement attack, since these cells often lack an important membrane-bound complement inhibitor protectin (CD59) (Seifert *et al.* 1992), whereas CRP, apoptotic cells and modified lipids represent potential activators of the complement system in the lesions (see the section *Complement activators in atherosclerotic lesions*).

Complement activators in atherosclerotic lesions

During atherogenesis, various structures that are capable of activating complement are either generated or deposited in the arterial wall. These include immune complexes containing IgM or IgG, pentraxins CRP and PTX3, and also modified lipids. In addition, other potential complement activators in the lesions include cholesterol crystals (Seifert & Kazatchkine 1987), apoptotic cells (Mevorach *et al.* 1998), and cell debris (Pinckard *et al.* 1975).

Immune complexes are traditionally regarded as activators of the classical complement pathway, but they have recently also been shown to increase the activation of the alternative complement pathway by stabilizing C3 convertase (Ji *et al.* 2002). Since the arterial wall is known to contain various (auto)antigens, against which a humoral immune response is generated, it is likely that atherosclerotic lesions contain at least some immune complexes. Indeed, both of the immunoglobulins G and M have been extracted and immunohistochemically detected in atherosclerotic arterial intima, and part of the extracted IgG was found to be tissue-bound, suggesting that it was in the form of immune complexes (Hollander *et al.* 1979; Vlaicu *et al.* 1985b). In addition, lipoprotein-containing immune complexes have been isolated from atherosclerotic lesions (Ylä-Herttuala *et al.* 1994), and very recently autoimmune oxLDL-IgG immune complexes were verified to be able to activate the classical complement pathway (Saad *et al.* 2006).

CRP is a pentameric acute-phase protein that has been shown to activate the classical complement pathway both *in vitro* (Siegel *et al.* 1974) and *in vivo* (Wolbink *et al.* 1996). However, in contrast to complement activation by other classical pathway activators, CRP-initiated activation inefficiently generates C5b-9, which is likely to result from the recruitment of complement inhibitor factor H by CRP (Jarva *et al.* 1999). In contrast, CRP-initiated activation efficiently coats target structures with complement-derived opsonins. In regard to atherogenesis, CRP as well as another complement-activating pentraxin, pentraxin 3 (PTX3), have been detected in atherosclerotic lesions (Rolph *et al.* 2002). In the arterial wall, CRP binds to various ligands such as enzymatically modified lipids and apoptotic cells, activates the classical complement pathway, and may thus promote the uptake of target structures by phagocytes via either FcγRs or complement receptors (Bhakdi *et al.* 1999; Gershov *et al.* 2000). Regarding complement activation, monomeric, modified CRP is also of interest. This modified form CRP has been shown to be present in the arterial wall and it has suggested to be produced from native, pentameric CRP due to the low pH at the sites of inflammation, for instance (Diehl *et al.* 2000). Regarding complement activation, a recent study showed that fluid-phase mCRP inhibited complement activation, possibly by binding to the collagen-like region of C1q and thus preventing its binding to other complement activators. Interestingly, however, when mCRP was immobilized to modified lipoproteins, for instance, it activated the early classical complement pathway (Ji *et al.* 2006).

During atherogenesis, intimal lipoproteins undergo modifications, which allow them to activate the complement system. Indeed, lipids isolated from human atherosclerotic lesions are able to activate the alternative complement pathway (Seifert *et al.* 1990), and lipids complexed with C5b-9 have been extracted from the intima (Seifert *et al.* 1990). In addition, enzymatic modification of LDL by trypsin, cholesterol esterase, and neuraminidase converts LDL to particles with properties akin to those extracted from atherosclerotic lesions, including the capacity to activate the complement to completion via the alternative pathway (Bhakdi *et al.* 1995). However, at low concentrations of E-LDL, CRP bound to E-LDL can activate the classical complement pathway and, likely via recruitment of factor H, stops the activation before the generation of C5b-9 (Bhakdi *et al.* 1999; Bhakdi *et al.* 2004). Accordingly, in the

very early stages of atherosclerosis, E-LDL showed only little colocalization with C5b-9 (Bhakdi *et al.* 2004), whereas colocalization was obvious in more advanced lesions (Torzewski *et al.* 1998b).

Complement effectors

There are three main mechanisms through which complement exerts its effects on inflammation and the immune response. First, complement activation generates large numbers of split products of complement proteins and their inactivation products (such as C4b, C3b and iC3b), which can bind covalently to target structures and promote their removal by phagocytes bearing receptors for complement proteins. Second, small fragments called anaphylatoxins (C4a, C3a, C5a) are generated in the cleavage of complement proteins. These peptides are chemokines and can also activate various cells. Third, the terminal membrane-attack complex (C5b-9) can form a pore-like structure on the bacterial/cell surface and thus destroy it, but it is important to note that, at non-lethal "sublytic" concentrations, C5b-9 does not kill the target cells, but rather elicits many pro-inflammatory effects in them.

Opsonins

One of the most important functions of complement is to facilitate the uptake of foreign particles and pathogens by phagocytic cells. In addition, the same mechanisms act in the clean-up of tissues from, for instance, cell debris. This process involves the coating of target particles by opsonins such as split products of C4 and C3, of which C3b and iC3b are the most important. The receptor for C3b and C4b is CR1 (CD35), and the receptors for iC3b are CR3 (CD11b/CD18) and CR4 (CD11c/CD18). In atherosclerotic lesions, CR1 and CR3 are both expressed at least by macrophages (Seifert & Hansson 1989; Saito *et al.* 1992). In addition to cell debris, opsonization plays a role in the clearance of apoptotic cells. Apoptotic cells can activate both the classical and alternative complement pathways resulting in the deposition of iC3b on their surfaces, followed by phagocytosis of dying cells via CR3 with a minimal inflammatory reaction (Mevorach *et al.* 1998; Gershov *et al.* 2000). In addition, opsonization of antigens can also modulate the adaptive immune response against them. Thus, it was recently found that opsonization with C3d markedly increased, and opsonization with C3b decreased the immunogenity of various antigens, presumably acting via complement receptors present on B lymphocytes, dendritic cells, and T cells (reviewed by Morgan *et al.* 2005). Furthermore, a recent paper suggested that the formation of C3b and other C3-derived molecules could at least partly explain the antiatherogenic effect of intravenous immunoglobulin injections in $LDLR^{-/-}$ mice (Persson *et al.* 2005).

Anaphylatoxins

During complement activation, the proteolytic cleavage of C4, C3 and C5 results in the generation of short-lived anaphylatoxins C4a, C3a and C5a, respectively. Of these, C4a has been shown to possess only weak activity, whereas C3a and C5a are potent inflammatory mediators. Once generated, C3a and C5a exert their biological effects through specific seven transmembrane domain G-protein-coupled receptors, which have been found to be expressed in myeloid cells such as monocytes, neutrophils and mast cells (Martin *et al.* 1997; Zwirner *et al.* 1999; Fureder *et al.* 1995) as well as in various non-myeloid cells such as neurons of the central nervous system (Davoust *et al.* 1999; Gasque *et al.* 1998; Gasque *et al.* 1997), tubular cells in the kidneys (Braun *et al.* 2004; Zahedi *et al.* 2000) and endothelium and smooth muscle cells in the lungs (Fregonese *et al.* 2005). The expression of the two anaphylatoxin receptors (C3aR and C5aR) is greatly increased during inflammation, as has been demonstrated in the inflamed human central nervous system (Gasque *et al.* 1998).

Both anaphylatoxins are potent mediators of inflammation, but they also exert immunomodulatory effects on both innate and adaptive immune response. Thus, C3a and C5a are chemotactic for monocytes (Arend *et al.* 1989; Zwirner *et al.* 1998b) and mast cells (Hartmann *et al.* 1997), cause respiratory burst (Fischer *et al.* 1995) and secretion of proinflammatory cytokines from macrophages (Takabayashi *et al.* 1998; Takabayashi *et al.* 1996; Goodman *et al.* 1982; Ember *et al.* 1994), and trigger mast cell degranulation (Schulman *et al.* 1988; el-Lati *et al.* 1994). Furthermore, C5a has been shown to cause upregulation of adhesion molecules on endothelial cells (Foreman *et al.* 1996; Albrecht *et al.* 2004), and both C3a and C5a have been shown to induce expression of proinflammatory cytokines in cultured human umbilical vein endothelial cells (Albrecht *et al.* 2004; Monsinjon *et al.* 2003). In addition, activation of mast cells and macrophages through C5aR has been shown to regulate the expression of Fcγ receptors and thus to play a causal role in experimental immune complex-induced disease (Bozic *et al.* 1996; Hopken *et al.* 1997; Godau *et al.* 2004; Baumann *et al.* 2001; Shushakova *et al.* 2002). Although C3a and C5a share many similar functions, they seem to have to some extent opposing effects on the regulation of adaptive immunity. Thus, C5a is chemoattractant for T lymphocytes (Nataf *et al.* 1999; Tsuji *et al.* 2000), potentiates T cell proliferation (Morgan *et al.* 1983), and drives the Th1-type immune response that has been suggested to be proatherogenic. In contrast, C3a has been shown to mediate immunosuppressive effects (Morgan *et al.* 1982) and also to drive the Th2-type immune response (Hawlisch *et al.* 2004) that has been suggested to promote antiatherogenic effects.

Membrane-attack complex

Activation of the terminal complement pathway, which leads to the assembly of the membrane-attack complex C5b-9 and forms functional pores in the cell membrane, may result in cell swelling and subsequent cell lysis. A single functional channel is sufficient to cause lysis of an erythrocyte, but nucleated cells are more resistant, since at low “sublytic” concentration of C5b-9, they can escape lysis by exocytosing and endocytosing C5b-9. However, these non-lethal “sublytic” concentrations of C5b-9 may affect cell function and mediate signaling via various pathways, e.g. by inducing the influx of calcium into cell (Seeger *et al.* 1986). Moreover, C5b-9 directly activates signaling pathways that involve NFκB and cdk4/cdk2, which are known to activate a set of pro-inflammatory genes, and DNA synthesis and cell proliferation, respectively (Kilgore *et al.* 1997; Niculescu & Rus 1999). Accordingly, *in vitro* studies have demonstrated that sublytic concentrations of C5b-9 have many effects that may increase inflammation and also affect the stability of atherosclerotic lesion. Thus, sublytic concentrations of C5b-9 upregulate the expression of the adhesion molecules ICAM-1 and VCAM-1 and E-selectin by endothelial cells (Tedesco *et al.* 1997; Kilgore *et al.* 1995). In addition, sublytic concentration of C5b-9 induce secretion of the growth factors bFGF and PDGF (Benzaquen *et al.* 1994) and the chemotactic molecules interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) from endothelial cells (Kilgore *et al.* 1997). Furthermore, the assembly of C5b-9 on smooth muscle cells leads to the release of monocyte chemotactic protein-1 (Torzewski *et al.* 1996) and induces SMC proliferation (Niculescu *et al.* 1999). C5b-9 may also protect vascular smooth muscle cells from apoptosis by inducing secretion of insulin-like growth factor-1 (Zwaka *et al.* 2003). However, at higher concentrations, C5b-9 has also been shown to induce caspase activation and apoptosis (Nauta *et al.* 2002a), and it has been shown to colocalize with markers of apoptosis in atherosclerotic lesions (Niculescu *et al.* 2004), suggesting that C5b-9 may be involved in the induction of apoptosis of vascular cells.

Complement regulators in atherosclerotic lesions

The complement system is regulated at almost all the levels of activation by both soluble and membrane-bound regulators. This is of great importance, since it prevents complement from attacking host cells and protects the tissues of the host from being damaged by uncontrolled complement activation. Various complement regulatory proteins have also been shown to be present in atherosclerotic lesions (Seifert & Hansson 1989). In addition, there is evidence that complement regulatory proteins are actively expressed in arteries, and their expression level is similar in both normal and atherosclerotic vessel wall (Yasojima *et al.* 2001a). Complement regulators are listed in Table 2, and the sites of their action are shown in Fig. 3. Table 2 also lists the studies that have demonstrated the presence of complement regulators in atherosclerotic lesions.

Table 2. Complement regulators, their function, and their presence in atherosclerotic lesions

Regulatory protein	General function	Demonstrated to be present in atherosclerotic lesions by:
Soluble regulators		
C1-inhibitor	Binds and inactivates C1r and C1s	Seifert & Hansson 1989
C4b-binding protein	Accelerates decay and inhibits CP C3 convertase, cofactor for factor I	Kimoto *et al.* 1996
Factor H	Accelerates decay and inhibits AP C3 convertase, cofactor for factor I	Seifert & Hansson 1989
Factor I	Cleaves and inactivates C3b and C4b in the presence of cofactor	
Properdin	Stabilizes AP C3 convertase	Seifert & Hansson 1989
S protein	Prevents assembly of C5b-9	Niculescu *et al.* 1987b
Clusterin	Prevents assembly of C5b-9	Ishikawa *et al.* 1998
Membrane-bound regulators		
DAF (CD55)	Accelerates decay of AP and CP C3 and C5 convertases	Seifert & Hansson 1989
MCP (CD46)	Cofactor for factor I	
CR1 (CD35)	Accelerates decay of AP and CP C3 and C5 convertases, cofactor for factor I	Saito *et al.* 1992
Protectin (CD59)	Prevents assembly of C5b-9	Seifert *et al.* 1992

AP, alternative pathway; CP, classical pathway; DAF, decay-accelerating factor; MCP, membrane cofactor protein; CR1, complement receptor 1

The immunohistochemical studies showing the presence of regulators in atherosclerotic lesions failed to provide evidence for the regulatory activity of these components in complement activation in lesions. Thus, Seifert and coworkers were unable to demonstrate any obvious spatial relation between C5b-9 complexes and any of the complement regulatory proteins studied (CR1, C1-INH, factor H, properdin, DAF) (Seifert & Hansson 1989). Similarly, terminal C5b-9 complexes were present in areas with and without CD59 (Seifert *et al.* 1992) and DAF (Niculescu *et al.* 1990). However, the partial colocalization of S-protein/vitronectin and C5b-9 suggested that a fraction of C5b-9 may have been inactivated/solubilized by S-protein/vitronectin (Niculescu *et al.* 1987c). As this thesis concentrates on the role C4b-binding protein and factor H in the regulation of complement

activation in atherosclerotic lesions, only these two regulators will be described in greater detail.

C4b-binding protein (C4bp) is a key inhibitory molecule for the classical and lectin pathways of complement. The average plasma concentration of C4bp is ~260 nM (150 µg/ml) (Griffin *et al.* 1992), but since it is an acute-phase protein, its levels may increase up to 4-fold during inflammation, infection, and tissue damage (Saeki *et al.* 1989). C4bp is a 500 kDa molecule, which consists of six or seven α-chains and one β-chain. The α-chains are built up of eight short consensus repeat (SCR) domains, and the β-chain contains three such SCR modules (Hillarp & Dahlbäck 1988). The chains are linked together by disulfide bridges at their C-terminal domains, and C4bp therefore appears as a spider-like structure in electron microscopy (Dahlbäck *et al.* 1983). C4bp inhibits the classical complement pathway by preventing the assembly and accelerating the decay of the C4b2a complex, and acting as a cofactor for factor I in the cleavage and inactivation of C4b (Gigli *et al.* 1979). The C4b-binding site has been localized to the SCRs 1–3 of the α-chains of C4bp, and the same area also harbors the binding sites for heparin (Blom *et al.* 2001). The majority of C4bp circulates in complex with protein S, which is part of the anticoagulant system. Upon the formation of this complex, the anticoagulant activity of protein S is lost (Dahlbäck 1986), but the complement inhibitory properties of C4bp remain unaffected. In atherosclerotic aortas, C4bp has been found to locate diffusely in the intima, with more intensive staining in foam cells and the necrotic core (Kimoto *et al.* 1996). In addition, the intimal cells of both normal and atherosclerotic aortic wall were found to express C4bp (Yasojima *et al.* 2001a).

Factor H is a key inhibitor of activation by the alternative pathway. Factor H is a single-chain glycoprotein (155 kDa) that is present in plasma at concentrations ranging from about 100 µg/ml to about 600 µg/ml. Factor H is composed of 20 repetitive short consensus repeat domains (SCR) (Zipfel *et al.* 1999), which form a string-like structure. Factor H binds C3b and thus inhibits the assembly and promotes the decay of alternative pathway C3 convertase and further acts as a co-factor for factor I in the proteolytic cleavage of C3b (Whaley & Ruddy 1976; Weiler *et al.* 1976; Pangburn *et al.* 1977). Factor H regulates complement both in the fluid phase and on cellular surfaces, but the inactivation of surface-bound C3b by factor H is dependent on the presence of sialic acids or other polyanionic molecules on the surfaces of intact human cells (Fearon 1978). In addition to containing three distinct binding sites for C3b (Jokiranta *et al.* 2000), factor H also contains three heparin-binding sites (Blackmore *et al.* 1996; Blackmore *et al.* 1998; Pangburn *et al.* 1991). Previous studies detected factor H in atherosclerotic but not in non-atherosclerotic intimas, where it was located on connective-tissue fibers in a granular, thread-like pattern (Seifert & Hansson 1989).

Complement-deficient animals and atherosclerosis

Experimental studies with complement-deficient animals have been made to elucidate the role of the complement system in atherogenesis, but the results have been controversial. As early as 1970, Geertinger and Sørensen showed that the formation of vitamin D-induced atherosclerotic-like lesions in rats was diminished when the complement system was depleted by using zymosan (Geertinger & Sørensen 1970). Later, the same group observed that an atherogenic diet induced less atherosclerosis in rabbits deficient in C6 than in controls with a fully functional complement system (Geertinger & Sørensen H 1977). These results were confirmed by a similar experimental setup twenty years later (Schmiedt *et al.* 1998). Similarly, an anticomplement agent K-76 COONa was found to suppress the development of atherosclerosis in cholesterol-fed rabbits without affecting serum cholesterol levels (Saito *et*

al. 1990). In addition, injections of a complement inhibitor, vaccinia virus complement control protein, led to significantly reduced lesion size in C57Bl/6 mice fed high-fat diet (Thorbjornsdottir *et al.* 2005) However, contrasting results were obtained in C5 deficient $apoE^{-/-}$ mice, which showed a similar extent of atherosclerotic lesions compared to complement-competent control mice (Patel *et al.* 2001). More recently, two studies investigated the role of C3 deficiency in atherosclerosis. A study by Buono and coworkers showed that, in $LDLR^{-/-}$ mice, C3 deficiency leads to larger lipid-positive areas in *en face* preparations of descending aorta, but not in the aortic root. In addition, lesions contained increased numbers of macrophages but decreased quantities of collagen and smooth muscle cells, suggesting that maturation was impared in the absence of C3 (Buono *et al.* 2002). Similarly, the study by Persson and coworkers reported increased atherosclerosis in the descending aortas of C3 deficient $LDLR^{-/-}apoE^{-/-}$ mice, while no qualitative differences were observed between the lesions in C3 deficient and complement-competent control mice (Persson *et al.* 2004). Of interest, in the latter study C3 deficient mice had a more atherogenic lipid profile than control mice, which probably resulted from defective triglyceride synthesis by adipocytes because of the absence of C3-derived acylation-stimulating protein (ASP). In contrast, deficiency of factor B affected neither lipid levels nor lesion size (Persson *et al.* 2004). These results, together with a recent study demonstrating that C3 deficiency abolishes the antiatherogenic effect of intravenous immunoglobulin treatment (Persson *et al.* 2005), suggest that the activation of the early complement pathway with generation of opsonins may exert atheroprotective effects, whereas the terminal pathway with C5b-9 formation is more proatherogenic.

2.4 Adaptive immunity in atherosclerosis

The adaptive immune response is based on the antigen-specific reactions proceeding through T and B lymphocytes. In contrast to innate immunity, this type of response is slow and may take days to weeks to develop. However, the adaptive immune response has a memory, and subsequent exposure to the antigen leads to a more vigorous and rapid response, which reflects the pre-existence of a clonally expanded population of antigen-specific lymphocytes. The capacity of adaptive immunity to respond specifically to a wide variety of different antigens relies on the somatic rearrangement processes in progenitor cells, which can potentially results in the generation of approximately 10^{16} different T cell receptors (TCR) and 10^{11} different immunoglobulin molecules, which are used as B cell receptors. However, the actual number of these receptors in individual is only approximately 10^{7}-10^{11} (Cellular and Molecular Immunology, 5^{th} Edition, 2005 by Abbas *et al.*). T cells recognize the complex of antigenic peptide in association with self MHC molecules and require additional co-stimulatory signals to become activated and to exert their effects. Cytotoxic CD8+ lymphocytes act directly by attacking cells that present their specific antigen on MHC class I molecules, whereas helper CD4+ T cells recognize antigens on MHC class II molecules and provide help for other immune cells either by producing cytokines or by direct cell-cell interactions. In contrast, B cells can recognize their specific antigens directly with their surface-bound IgM molecules, and upon further help from T cells, they start producing antibodies of similar specificity. Atherosclerotic lesions have been shown to contain cells of adaptive immunity and antibodies as well as cytokines produced predominantly by these cells, and animal studies have suggested that adaptive immunity plays a role in atherogenesis (see the section *Effects of immune system in atherogenesis*). The players of adaptive immunity in atherosclerotic lesions are summarized in Figure 4.

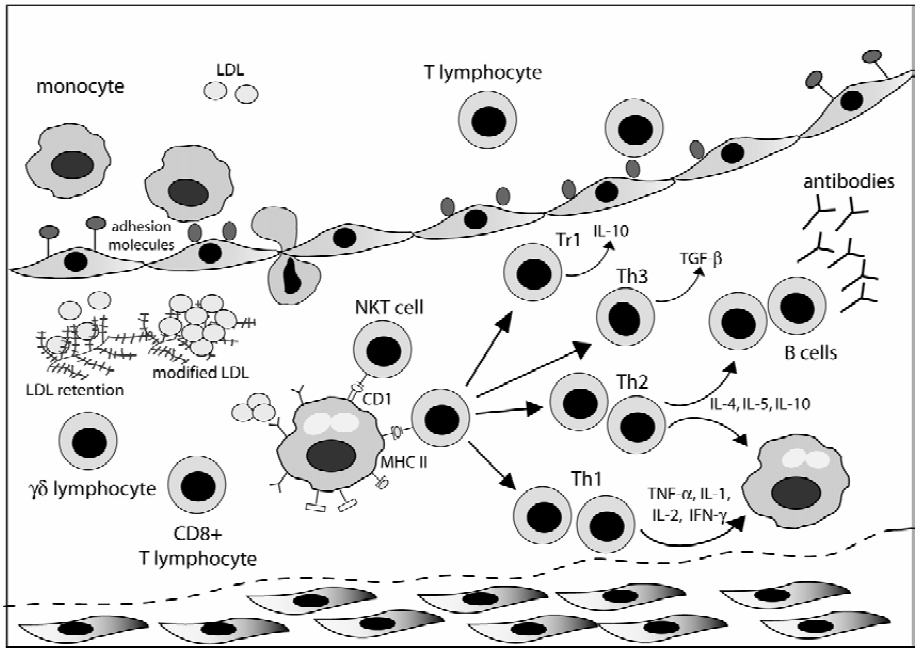

Figure 4. Schematic representation of the players of the adaptive immune system in atherosclerotic lesions. Antigen-presenting cells, notably macrophages and dendritic cells, present antigens on the surface MHC class II molecules for recognition by cells of adaptive immunity, i.e. lymphocytes. As a result, both cellular and humoral (cytokines, antibody production) immune responses toward antigen are initiated. Adapted from Shah *et al.* (2004).

2.4.1 T lymphocytes

T lymphocytes enter the arterial wall early in atherogenesis, and these cells can therefore be observed in atherosclerotic lesions at all stages of lesion development (Jonasson *et al.* 1986; van der Wal *et al.* 1989). In advanced atherosclerotic lesions, about 5 to 20% of cells are T lymphocytes (Jonasson *et al.* 1986; Hansson *et al.* 1989), of which the majority are CD4+ helper T cells bearing αβ T cell receptor. A substantial proportion of T cells also bear markers such as IL-2 receptor, MHC class II, CD45RO, or very late antigen-1 (VLA-1), suggesting that these cells are in an activated state (Hansson *et al.* 1989; van der Wal *et al.* 1989; Stemme *et al.* 1992) and may have become stimulated by local antigens. Indeed, upon encounter with and recognition of an antigen, T cells may become activated and proliferate, giving rise to a clone of cells with identical specificities. Interestingly, the lesions of apoE-deficient mice have shown oligoclonal expansion of T cells, indicating that the T cell population has expanded due to local antigenic stimulation (Paulsson *et al.* 2000). One of the clones expressed TCR-Vβ6, a receptor type that is also expressed by T cells recognizing oxLDL. In contrast, such clonal expansion could not be detected in human advanced atherosclerotic lesions, a result that might be explained by the presence of a more chronic type of inflammation in older plaques (Stemme *et al.* 1991; Swanson *et al.* 1994; Oksenberg *et al.* 1997). However, to support the role of intimal antigens in the local activation of adaptive immunity, T cells reactive with oxLDL (Stemme *et al.* 1995), HSPs (Benagiano *et al.* 2005), EBV (Boer de *et al.* 2005), and chlamydial antigens (Mosorin *et al.* 2000; Boer de *et al.* 2000) have been isolated from human atherosclerotic plaques. It is of special interest, that the percentage of recently activated T lymphocytes was found to be increased in the culprit lesions of patients with acute coronary syndromes (Hosono *et al.* 2003), and that the T cells of unstable plaques showed signs of specific, antigen-driven activation (De Palma *et al.* 2006).

In addition, in acute coronary syndromes, the peripheral blood lymphocyte response is directed against plaque antigens (Caligiuri *et al.* 2000). These results suggest that activation of T lymphocytes may play a role in the destabilization of plaques. Accordingly, most studies on experimental animals suggest that, as a group, T cells are proatherogenic. Thus, the absence of CD4+ T cells has been shown to reduce lesion formation in apoE$^{-/-}$ mice (Zhou *et al.* 2005), while transfer of CD4+ T cells aggravates atherosclerosis in immunodeficient apoE$^{-/-}$ mice (Zhou *et al.* 2000).

CD4+ T lymphocytes have been divided into subgroups based on the cytokines they secrete, and these subgroups have been suggested to play somewhat opposing roles in the pathogenesis of atherosclerosis. Thus, Th1 cells, which produce cytokines that promote cell-mediated immunity (IFN-γ, IL-1, TNF-α, and IL-2), have been regarded to promote atherogenesis. In contrast, Th2 cells, which secrete cytokines involved in antibody production (IL-4, IL-5, IL-10, and IL-13), have been suggested also to mediate anti-atherogenic effects. Recently, a third subgroup of regulatory T cells has gained much attention in atherogenesis. These cells are further divided into IL-10-producing Tr1 cells and TGF-β-producing Th3 cells, and they exert many immunoregulatory functions, play a critical role in the maintenance of immunological tolerance, and hence have also been shown to mediate anti-atherogenic effects.

Th1 response

The Th1 type of response, favoring a proinflammatory cell-mediated immune reaction, is suggested to prevail in atherosclerotic lesions. Thus, lesional T cells, especially in early lesions, have largely the properties of the Th1 subtype, and a large proportion of human atherosclerotic lesions contain IL-2 and IFN-γ, two important Th1-derived cytokines (Frostegård *et al.* 1999). Accordingly, various factors that promote the Th1 type immune response, e.g. IL-12 (Uyemura *et al.* 1996), IL-18 (Mallat *et al.* 2001a), and osteopontin (O'Brien *et al.* 1994) have been demonstrated in lesions. The Th1 type immune response has been suggested to promote atherogenesis. Thus, Th1 cytokines have been shown to activate macrophages, induce endothelial dysfunction, and promote plaque destabilization by inhibiting SMC proliferation and collagen synthesis, by inducing SMC apoptosis, and by increasing the secretion of matrix-degrading enzymes (Raines & Ferri 2005; Daugherty *et al.* 2005; Østerud & Bjørklid 2003; Geng *et al.* 1996). Accordingly, animal studies have supported this pro-atherogenic role of the Th1 type response. Administration of exogenous IL-12 (Lee *et al.* 1999), IL-18 (Whitman *et al.* 2002b), or IFN-γ (Whitman *et al.* 2000) has been shown to lead to increased atherosclerosis in apoE$^{-/-}$ mice. In this mouse model, IL-18 has also been shown to induce structural changes, such as decreased collagen content of plaques, which increases plaque instability (Mallat *et al.* 2001b; Nooijer de *et al.* 2004). In accordance with these results, apoE$^{-/-}$ mice with genetic deficiency of IL-18 (Elhage *et al.* 2003), IFN-γ (Whitman *et al.* 2002a) or TNF-α (Brånen *et al.* 2004) showed decreased atherosclerosis. Futhermore, treatment of apoE$^{-/-}$ mice with pentoxifyllin, which inhibits Th1 differentiation, decreased lesion formation (Laurat *et al.* 2001), and promotion of the Th1 type response by genetic alterations increased fatty streak formation in BALB7c mice (Huber *et al.* 2001).

Th2 response

In contrast to Th1 cytokines, Th2 cytokines are scarce in human atherosclerotic plaques (Frostegård *et al.* 1999; Uyemura *et al.* 1996; Mallat *et al.* 1999b). However, based on the results of animal studies, it has been suggested that a shift toward the Th2 type response may

occur during the progression of atherosclerosis. Thus, severe hypercholesterolemia has been shown to shift the initial Th1 type autoimmune response toward the Th2 response in apoE$^{-/-}$ mice, as reflected by the increased levels of Th2-dependent anti-oxLDL IgG_1 antibodies, the increased number of IL-4 and IL-10-producing T cells in the spleen, and the infiltration of IL-4-producing T cells into atherosclerotic lesions (Zhou *et al.* 1998). Many studies have suggested an atheroprotective role for the Th2 response, an effect mediated at least partly by suppression of the Th1 type pro-inflammatory response (Uyemura *et al.* 1996). Indeed, in atherosclerotic lesions, areas containing the Th2 cytokine IL-10 show reduced apoptosis and expression of inflammatory genes, reflecting weakening of the Th1 response (Mallat *et al.* 1999b). Furthermore, experiments with mice models have shown that overexpression of IL-10 by T lymphocytes leads to decreased atherosclerosis, and accordingly, IL-10 deficiency leads to increased atherosclerosis (Pinderski Oslund *et al.* 1999; Pinderski *et al.* 2002; Mallat *et al.* 1999a; Caligiuri *et al.* 2003). In addition, induction of the Th2 type of immune response has also been suggested to play a role in the atheroprotection observed in apoE$^{-/-}$ in response to immunization with MDA-LDL (Binder *et al.* 2004; Fredrikson *et al.* 2005). However, some data indicates that the Th2 type response could also mediate proatherogenic effects. Indeed, IL-4 has been shown to regulate various processes that can be regarded as potentially proatherogenic, such as monocyte recruitment and lipoprotein modification, and genetic deficiency of IL-4 is associated with decreased atherosclerosis in LDLR$^{-/-}$ mice (King *et al.* 2002).

Regulatory T lymphocytes

Regulatory T cells (Tregs) play an important role in the induction and maintenance of immunological tolerance. The Th3 and Tr1 types of Tregs have been suggested to be of special importance in acquired tolerance to oral antigens as well as altered self-peptides. These cells have been shown to suppress both the Th1 and Th2 of types of response predominantly via the production of two anti-inflammatory cytokines IL-10 and TGF-β (reviewed by Cottrez & Groux 2004). Both cytokines are present in atherosclerotic lesions (Mallat *et al.* 1999b; Frostegård *et al.* 1999) and have been shown to mediate antiatherogenic effects. Thus, TGF-β deactivates macrophages, inhibits the maturation of dendritic cells, inhibits T cell proliferation and differentiation, reduces the expression of adhesion molecules, and promotes matrix deposition (reviewed by Mallat & Tedgui 2002). Indeed, the inhibition of TGF-β signaling in mouse models of atherosclerosis either systemically or in T cells led to the generation of unstable plaques with large lipid cores, extensive inflammatory infiltrates, and a decreased collagen content (Mallat *et al.* 2001c; Lutgens *et al.* 2002; Gojova *et al.* 2003; Robertson *et al.* 2003). In addition, transfer of Tr1 cells with their cognate antigen reduced atherosclerosis in apoE$^{-/-}$ mice with a concomitant increase in the level of IL-10 but a decrease in the number of macrophages and T cells in the lesions (Mallat *et al.* 2003). Immunological tolerance mediated by Tregs may also explain the observed immune suppression and reduction in atherosclerosis in LDLR$^{-/-}$ mice after oral/nasal feeding of HSP-65 (Harats *et al.* 2002; Maron *et al.* 2002). In addition to antigen-induced adaptive regulatory T cells, endogenous natural CD4+CD25+ regulatory T cells may play a role in the control of atherosclerosis. Accordingly, very recent paper showed that these natural regulatory T cells are efficient inhibitors of atherosclerosis in mice (Ait-Oufella *et al.* 2006).

CD8+, γδ and NKT lymphocytes

In addition, atherosclerotic lesions have been shown to contain minor populations of other types of T lymphocytes. Some cytotoxic CD8+ cells are present in the intima throughout lesion development (Jonasson *et al.* 1986). However, since CD8+ deficiency did not affect

lesion size in apoE$^{-/-}$ mice, they have been suggested to play only a minor role in atherogenesis (Elhage *et al.* 2004). Similarly, γδ T lymphocytes, which express γδ TCR (in contrast to the more common αβ TCR), have also been detected in atherosclerotic lesions (Kleindienst *et al.* 1993), and their absence only marginally decreased atherosclerosis in apoE$^{-/-}$ mice (Elhage *et al.* 2004). In contrast, NKT lymphocytes, which share characteristics of both NK cells of innate immunity and T cells of adaptive immunity, have been suggested to play a role in atherogenesis. These cells recognize lipid and glycolipid antigens presented on CD1 molecules on antigen-presenting cells (Beckman *et al.* 1994), and upon activation, they can produce large amounts of cytokines of both the Th1 and the Th2 type. CD1-positive cells have been detected in human atherosclerotic lesions (Melian *et al.* 1999), and NKT lymphocytes have been shown to accumulate in atherosclerosis-prone areas of the aortic arch in LDLR$^{-/-}$ mice (Aslanian *et al.* 2005). Of interest, animal studies have demonstrated that C1d-dependent activation of NKT cells aggravates atherosclerosis (Major *et al.* 2004; Tupin *et al.* 2004; Nakai *et al.* 2004; Aslanian *et al.* 2005).

2.4.2 B lymphocytes

B lymphocytes give rise to antibody-producing plasma cells. However, this process of antibody production typically requires assistance from helper T cells. Thus, upon recognition of the specific antigen by surface IgM, B cells internalize the antigen, process it, and re-express it on their surface MHC class II molecules for recognition by Th cells with similar specificity. Upon this interaction, Th cells become activated and provide additional signals such as costimulatory molecules and cytokines to promote B cell differentiation, somatic hypermutation, and immunoglobulin class switching. In addition, a subpopulation of B cells express CD1d, which suggests that they might be involved in the presentation of lipid antigens to NKT cells (Mizoguchi *et al.* 2002). B cells have been detected in low numbers in both human and murine atherosclerotic lesions (Millonig *et al.* 2002; Zhou & Hansson 1999), but these cells are more numerous in the adventitia and periadventitial tissue (Aubry *et al.* 2004). Furthermore, lesions of Watanabe hyperlipidemic rabbits were shown to contain clones of immunoglobulin-producing plasma cells (Sohma *et al.* 1995). Indeed, immunoglobulins have been identified in human atherosclerotic lesions throughout the process of lesion development (Vlaicu *et al.* 1985b; Ylä-Herttuala *et al.* 1994). Although it is likely that part of the antibodies have entered the intima from plasma, the presence of mRNAs for the κ light and γ heavy chain of IgG in rabbit lesions suggests that immunoglobulins are also synthesized locally in atherosclerotic plaques (Sohma *et al.* 1995). As a group, B cells have been suggested to play an atheroprotective role, possibly by affecting both the generation of protective antibodies and the immune network in a more general way. In support of this notion, removal of the spleen, which harbors a significant proportion of memory B cells and plays an important role in B cell maturation, significantly increased atherosclerosis in apoE$^{-/-}$ mice, and transfer of B cells from atherosclerotic apoE$^{-/-}$ mice reversed this effect and protected mice from advanced disease (Caligiuri *et al.* 2002). In addition, complete B cell deficiency increased both the early and late stages of atherosclerosis in LDLR$^{-/-}$ mice (Major *et al.* 2002). In this regard, of special interest and importance might be a subpopulation of B cells called B-1 cells, which belong to innate immunity, produce IgM-type natural antibodies, are abundant in spleen, and are reported to be the source of protective antibodies after immunization of mice with MDA-LDL (Binder *et al.* 2004). These antibodies are discussed in more detail in the section *B1 cells and natural antibodies*.

Table 3. Clinical studies correlating the levels of anti-oxLDL antibodies with cardiovascular disease

Reference	LDL modification	Type of antibody	Number of subjects	Measured parameter	Main results
Salonen *et al.* 1992	MDA, Cu^{2+}	IgG, IgM, IgA	30 cases, 30 controls (all healthy)	progression of carotid atherosclerosis (IMT)	IgG (MDA) ↑ in cases
Virella *et al.* 1993	Cu^{2+}	IgG	33 patients undergoing angiography, 64 healthy controls	coronary atherosclerosis	no difference between groups
Puurunen *et al.* 1994	MDA	IgG	135 cases, 135 controls (all dyslipidemic)	AMI during 5-year follow-up	IgG ↑ in cases
Maggi *et al.* 1994	Cu^{2+}, MDA	IgG, IgM	94 cases, 42 healthy controls	severe carotid atherosclerosis	IgG and IgM (Cu^{2+}, MDA) ↑ in cases
Armstrong *et al.* 1994	MDA	IgG	87 cases, 60 healthy controls	angiographically verified CAD	IgG ↑ in cases
Bergmark *et al.* 1995	Cu^{2+}	IgG, IgM, IgA	62 cases, 62 healthy controls	peripheral vascular disease	antibodies ↑ in cases
Boullier *et al.* 1995	MDA	IgG	70 cases, 70 healthy controls	angiographically verified CAD	no difference between groups
Bui *et al.* 1996	Cu^{2+}	IgG	25 cases, 38 healthy controls	angiographically-verified CAD	IgG ↑ in cases
Uusitupa *et al.* 1996	Cu^{2+}	IgG	91 NIDDM patients, 82 healthy persons	cardiovascular event and carotid IMT during 10-year follow up	no correlation between IgG and carotid IMT or CV events
van de Vijver *et al.* 1996	MDA	IgG	47 cases, 96 controls	severe CAD (>80%)	no correlation between IgG and severity of atherosclerosis
Wu *et al.* 1997	Cu^{2+}	IgG, IgM, IgA	119 cases, 138 controls (all healthy)	AMI during 10- to 20-year follow-up	IgG and IgA ↑ in cases
Lehtimäki *et al.* 1999	Cu^{2+}	IgG	58 cases, 34 controls	angiographically verified CAD	IgG ↑ in cases
Orchard *et al.* 1999	Cu^{2+}	IgG	patients with type I diabetes (49 cases, 49 controls)	AMI, angina, CAD death	IgG ↓ in cases
Fukumoto *et al.* 2000	Cu^{2+}	IgG	446 healthy persons	carotid atherosclerosis (IMT)	IgG ↓ related to increased IMT
Erkkilä *et al.* 2000	Cu^{2+}	IgG	101 cases, 314 controls (all with CAD)	AMI	IgG ↑ in cases (AMI) than in controls
Hulthe *et al.* 2001a	Cu^{2+}, MDA	IgG, IgM	388 healthy 58-year old men	carotid and femoral atherosclerosis (IMT)	IgG (Cu^{2+}) ↑ related to increased carotid IMT, IgG (Cu^{2+}) ↓ related to increased femoral IMT
Hulthe *et al.* 2001b	Cu^{2+}, MDA	IgG, IgM	102 hypercholesterolemic patients and 102 healthy controls	carotid atherosclerosis (IMT)	IgM (Cu^{2+}, MDA) ↓ related to increased IMT in controls
Dotevall *et al.* 2001	MDA	IgG, IgM	46 cases, 70 healthy or diabetic controls	AMI	IgG ↑ and IgM↓ in cases
Inoue *et al.* 2001	MDA	IgG	108 cases, 31 controls without CAD	angiographically verified CAD	IgG ↑ in cases
Meraviglia *et al.* 2002	Cu^{2+}, MDA	IgG	52 patients undergoing carotid endarterectomy	progression of carotid restenosis during 6 months follow-up	IgG and IgM (Cu^{2+}) ↑ related to progression
Inoue *et al.* 2002	MDA	IgG	39 cases, 25 controls	AMI	IgG ↑ in cases
McDowell *et al.* 2002	MDA	IgG	40 cases, 40 controls without CAD	angiographically verified CAD	no difference between the groups
Shoji *et al.* 2003	Cu^{2+}	IgG	103 patients with end-stage renal disease	carotid atherosclerosis (IMT)	IgG ↓ related to increased IMT
Fredrikson *et al.* 2003a	MDA-modified apoB-peptides	IgG, IgM	227 persons (78 cases, 149 controls)	carotid atherosclerosis (IMT) (AMI during follow-up)	IgM ↑ related to increased IMT, IgM ↑ in cases
Karvonen *et al.* 2003	Cu^{2+}, MDA	IgM, IgG	1022 middle-aged persons	carotid atherosclerosis (IMT)	IgM ↓ related to increased IMT
Rossi *et al.* 2003	MDA	IgG	529 patients undergoing coronary angiography	severity of coronary atherosclerosis	no correlation between IgG and severity of coronary atherosclerosis
Tornvall *et al.* 2003	Cu^{2+}, MDA, HOCl	IgG	88 cases, 88 healthy controls	AMI, prognosis of AMI patients during 11-year follow up	IgG (Cu^{2+}, MDA) ↑ in cases, no association between IgG and prognosis of cases
Fernandes *et al.* 2004	Cu^{2+}	IgG	15 cases, 15 controls with stable angina	unstable angina	IgG ↓ related to unstable angina
Su *et al.* 2005	Cu^{2+}, MDA	IgG, IgM	226 patients with hypertension	carotid atherosclerosis (IMT) during 4-year follow-up	IgM (Cu^{2+},MDA and PC) ↓ related to increased IMT

CAD, coronary artery disease; NIDDM, non-insulin-dependent diabetes mellitus; IMT, intima-media thickness, ↑ equals increase; ↓ equals decrease

OxLDL autoantibodies of adaptive immunity

Antibodies against oxLDL have been shown to be present in the circulation of both patients with atherosclerosis and healthy individuals. However, it is not known whether these antibodies play an active role in the pathogenesis of atherosclerosis, or whether they are markers of the extent of the diasease. Studies on atherosclerotic mice have suggested that the levels of anti-oxLDL antibodies reflect the extent of atherosclerosis (Palinski *et al.* 1995b). However, induction of their production by active immunization with differently generated types of oxLDL or passive addition of antibodies recognizing epitopes of aldehyde-modified LDL have been shown to significantly reduce lesion size (George *et al.* 1998b; Fredrikson *et al.* 2003b; Schiopu *et al.* 2004). The situation is even more complex in humans. Numerous clinical and epidemiological studies have quantified the levels of anti-oxLDL antibodies in order to assess their correlation with the severity and progression of atherosclerosis, but the results have been controversial. Thus, both IgM and IgG antibodies can be detected in healthy individuals as well as in patients with cardiovascular disease. In support of a protective or irrelevant role of oxLDL antibodies in atherogenesis, these antibodies are already present in healthy children with no cardiovascular risk even at concentrations much higher than in adults (Iughetti *et al.* 1999). Furthermore, various studies have shown an inverse association or no association between oxLDL antibodies, especially the IgM subtype, and cardiovascular disease (see Table 3). In contrast, several other studies have shown a positive correlation between the levels of anti-oxLDL antibodies and the risk factors of CVD, the severity of carotid atherosclerosis, and angiographically verified coronary artery disease and also demonstrated the predictive value of these antibodies in the progression of carotid atherosclerosis, the occurrence of myocardial infarction and cardiovascular mortality (see Table 3). To explain the controversial results, various explanations have been proposed. First, the antibody populations are heterogeneous, since the antibodies are generated due to the activation of both innate and adaptive immune responses. Second, the oxidation-specific epitopes they react with can be diverse. Third, individual variations in immune response and differences between study populations may have contributed to the results. Also differences in the methodology of determining antibody levels may have caused some variation, for instance, differences in the used antigen (Cu^{2+} vs. MDA-modification, the extent of oxidation, presence of immune complexes etc.) (reviewed by Virella & Lopes-Virella 2003). It is also worth remembering that an immune response to oxLDL may play different roles during the different stages of atherosclerosis.

2.5 Effects of immune response on atherogenesis

According to the current concepts, both innate and adaptive immune responses are activated in atherosclerosis. It is therefore intriguing to hypothesize that this disease process could be prevented by modulation of the immune response. During the last decade, genetic and other manipulations of the mouse immune system have provided us with increasing knowledge about the role of the immune system and its specific components in atherogenesis. In addition, comparison of the extent of atherosclerosis between patients with genetic or acquired immune defects and healthy controls has provided some data about the role of specific parts of the immunity in atherogenesis.

In humans, a study investigating the effects of congenital defects in humoral immunity on atherosclerosis failed to detect any association between IgA deficiency or common variable immunodeficiency (CVID) and acute myocardial infarction (Hammar *et al.* 1998). In contrast, HIV infection, which reduces the number of CD4+ helper T cells, has been associated with

cardiovascular diseases (Paton *et al.* 1993), and the extent of the disease has been suggested to correlate with the intensity of immunodeficiency (Constans *et al.* 1995). Moreover, current antiretroviral therapy used to treat HIV has been suggested to promote atherosclerosis largely by causing lipid alterations and insulin resistance (Barbaro 2003). Regarding the deficiencies of innate immunity, it has been shown that various deficiencies of the classical complement pathway proteins, i.e. C2 and C4, correlated with increased cardiovascular disease (Jönsson *et al.* 2005; Nityanand *et al.* 1999). This has been suggested to result at least partly from ineffective handling of apoptotic cells and immune complexes, thus also predisposing these patients to systemic lupus erythematosus (SLE). Of the other complement deficiencies, MBL deficiency has been associated with increased atherosclerosis and acute coronary events (Tsutsumi *et al.* 2005). Furthermore, TLR Asp299Gly polymorphism that attenuates TLR4 signaling is associated with decreased atherosclerosis and a reduced risk of acute coronary events in humans (Kiechl *et al.* 2002; Ameziane *et al.* 2003). However, some studies have failed to find such an association (Yang *et al.* 2003), and a recent report even suggested an increased risk of myocardial infarction in patients with Asp299Gly polymorphism (Edfeldt *et al.* 2004). Further support for the role of immune activation in the pathogenesis of atherosclerosis, is provided by the finding that patients with rheumatic diseases, i.e. rheumatoid arthritis and SLE, have a significantly increased risk of atherosclerosis compared to healthy controls, and this seems to be explained not only by traditional risk factors but also by other disease-related factors such as chronic inflammation and increased autoantibody titres (reviewed by Shoenfeld *et al.* 2005).

In mouse models of atherosclerosis, the use of various modern techniques, e.g. genetic manipulation and bone marrow transplantation, has allowed the researchers to investigate the role of specific parts of the immune system in atherosclerosis. In atherosclerosis research, two mouse models are widely used: apoE- and LDL-receptor-deficient mice. Targeted genetic deletion of apoE in mice leads to severe hypercholesterolemia and spontaneous atherosclerosis, whereas deletion of LDL receptor leads to atherosclerosis especially when the mice are fed a high-fat diet. In addition to mice, rabbits have been used in experimental research of atherosclerosis. Rabbits are very sensitive to high-cholesterol diets and develop rapidly severe dyslipidemia and atherosclerotic lesions. In addition, rabbits with a mutation in low density lipoprotein receptors, which develop spontaneous hypercholesterolemia (Watanabe Heritable Hyperlipidemic (WHHL) rabbits), have also been used in atherosclerosis research. The evidence from animal studies suggests that activation of the immune system may have both pro- and antiatherogenic effects, and these effects can be modulated by other factors such as gender and diet. Many of these studies have been discussed in relation to specific issues in separate chapters, and they are also summarized in Table 4. Therefore, they are only discussed here briefly.

In general, disruption of initial leukocyte infiltration into intima by genetic deletion of endothelial adhesion molecules, leukocyte integrins, or chemotactic molecules and their receptors have been reported to lead to decreased atherosclerosis. In regard to innate immunity, deficiency of pattern recognition receptors has been shown to decrease atherosclerosis, suggesting that these receptors mediate pro-atherogenic effects. In contrast, the role of CRP is controversial, and current data suggest only a minor, if any, role for it in murine atherosclerosis. However, it has been shown that transgenically expressed CRP can not activate the mouse complement system, and the consequences in mice are likely to differ significantly from those in humans (Reifenberg *et al.* 2005). In regard to complement itself, animal studies suggest that activation of the classical pathway, which is involved in opsonization, might be atheroprotective, whereas activation of the terminal pathway with

Table 4. Experimental studies on the effects of immune response on atherogenesis

Defect or treatment	Immunological effect	Animal /mouse strain	Atherosclerosis	Reference
Leukocyte infiltration and differentiation				
E-selectin deficiency		LDLR$^{-/-}$	↓	Dong *et al.* 1998
P-selectin deficiency		LDLR$^{-/-}$, apoE$^{-/-}$	↓	Dong *et al.* 1998; Dong *et al.* 2000; Johnson *et al.* 1997
VLA4 blocking		LDLR$^{-/-}$	↓	Shih *et al.* 1999
Hypomorphic VCAM-1		LDLR$^{-/-}$, apoE$^{-/-}$	↓	Cybulsky *et al.* 2001; Dansky *et al.* 2001
Leukotriene B4 antagonist		apoE$^{-/-}$, LDLR$^{-/-}$	↓	Aiello *et al.* 2002
MCP-1 deficiency		LDLR$^{-/-}$	↓	Gu *et al.* 1998
CCR2 deficiency		apoE$^{-/-}$	↓	Boring *et al.* 1998
CXCR3 deficiency		apoE$^{-/-}$	↓	Veillard *et al.* 2005
CX3CR1 deficiency		apoE$^{-/-}$	↓	Lesnik *et al.* 2003; Combadiere *et al.* 2003
CCR5 deficiency		apoE$^{-/-}$	↔	Kuziel *et al.* 2003
Met-RANTES	antagonism of CCR1, and -5	LDLR$^{-/-}$	↓	Veillard *et al.* 2004
Cells of the innate immunity				
M-CSF deficiency	lack of tissue macrophages	apoE$^{-/-}$	↓	Smith *et al.* 1995; Rajavashisth *et al.* 1998
Bone marrow transplant from Ly49a transgenic mice	lack of functional NK cells	LDLR$^{-/-}$	↓	Whitman *et al.* 2004
Pattern-recognition receptors				
SRA deficiency		apoE$^{-/-}$	↓	Suzuki *et al.* 1997
CD36 deficiency		apoE$^{-/-}$	↓	Febbraio *et al.* 2000
TLR4 deficiency		apoE$^{-/-}$	↔↓	Wright *et al.* 2000; Michelsen *et al.* 2004b
Acute-phase proteins				
CRP transgene		apoE$^{-/-}$	↑	Paul *et al.* 2004
CRP transgene		apoE$^{-/-}$	↔	Hirschfield *et al.* 2005
CRP transgene		apoE*3 Leiden	↔	Trion *et al.* 2005
CRP transgene		apoE$^{-/-}$	↔	Reifenberg *et al.* 2005
Complement system				
C6 deficiency		rabbit	↓	Schmiedt *et al.* 1998
C5 deficiency		apoE$^{-/-}$	↔	Patel *et al.* 2001
C3 deficiency		apoE$^{-/-}$/LDLR$^{-/-}$	↑	Persson *et al.* 2004
C3 deficiency		LDLR$^{-/-}$	↑ lipids, ↓ maturation	Buono *et al.* 2002
Vaccinia virus complement control protein injections	40-50% decrease in serum hemolytic activity (inhibition of complement activation)	C57Bl/6	↓	Thorbjornsdottir *et al.* 2005
T and B cells				
RAG1/RAG2 deficiency	T and B cell defect	apoE$^{-/-}$	↔	Dansky *et al.* 1997; Daugherty *et al.* 1997
RAG2 deficiency	T and B cell defect	apoE$^{-/-}$	↓	Reardon *et al.* 2001
RAG1 deficiency	T and B cell defect	LDLR$^{-/-}$	↓ (early)	Song *et al.* 2001
SCID/SCID	T and B cell defect	apoE$^{-/-}$	↓	Zhou *et al.* 2000
+ transfer of CD4+		apoE$^{-/-}$	↑	Zhou *et al.* 2000
CD4 deficiency		apoE$^{-/-}$	↓	Zhou *et al.* 2005
T-bet deficiency	↓ Th1 response	LDLR$^{-/-}$	↓	Buono *et al.* 2005
Pentoxifylline treatment	↓ Th1 response	apoE$^{-/-}$	↓	Laurat *et al.* 2001
IFNγR deficiency	↓ Th1 response, ↓ IFNγ effects	apoE$^{-/-}$	↓	Gupta *et al.* 1997
IFNγ deficiency	↓ Th1 response	apoE$^{-/-}$	↓	Whitman *et al.* 2002a
IL-18 deficiency	↓ Th1 response	apoE$^{-/-}$	↓	Elhage *et al.* 2003
TNF-α deficiency	↓ Th1 response	apoE$^{-/-}$	↓	Brånen *et al.* 2004
IFNγ treatment	↑ Th1 response	apoE$^{-/-}$	↑	Whitman *et al.* 2000
IL-12 treatment	↑ Th1 response	apoE$^{-/-}$	↑	Lee *et al.* 1999
IL-18 treatment	↑ Th1 response	LDLR$^{-/-}$	↑	Whitman *et al.* 2002b

Defect or treatment	Immunological effect	Animal /mouse strain	Atherosclerosis	Reference
IL-10 transgenic	↑ Th2 response	LDLR$^{-/-}$	↓	Pinderski *et al.* 2002
IL-4 deficiency in bone marrow-derived cells	↑ Th2 response	LDLR$^{-/-}$	↓	King *et al.* 2002
IL-5 deficiency in bone-marrow-derived cells	↓ T15/E06 antibodies	LDLR$^{-/-}$	↑	Binder *et al.* 2004
Inhibition of TGFβ signaling	↓ regulatory T cell response	apoE$^{-/-}$	↑ more unstable plaque phenotype	Mallat *et al.* 2001c; Lutgens *et al.* 2002
Injection of Treg cells	↑ regulatory T cell response	apoE$^{-/-}$	↓	Mallat *et al.* 2003
B cell deficiency		LDLR$^{-/-}$	↑	Major *et al.* 2002
Splenectomy	↓ B cell response	apoE$^{-/-}$	↑	Caligiuri *et al.* 2002
+ transfer of B cells		apoE$^{-/-}$	↓	Caligiuri *et al.* 2002
Others				
IgG injections	immunosuppression	apoE$^{-/-}$	↓	Nicoletti *et al.* 1998
IgG inj. (C3$^{+/+}$ / $^{-/-}$ mice)		LDLR$^{-/-}$	↓ (C3$^{+/+}$) ↔ (C3$^{-/-}$)	Persson *et al.* 2005
Disruption of CD40-CD40L interaction		apoE$^{-/-}$, LDLR$^{-/-}$	↓↔↔ more stable plaque phenotype	Mach *et al.* 1998; Schönbeck *et al.* 2000; Lutgens *et al.* 2000
B7-1/B7-2 deficiency	defects in T cell responses	LDLR$^{-/-}$	↓	Buono *et al.* 2004
Sirolimus injections	↓ Th1 and Th2 cytokines, except ↑ TGF-β	apoE$^{-/-}$	↓	Elloso *et al.* 2003
Immunization				
MDA-LDL s.c./i.m.	α-MDA-LDL IgG ↑	WHHL rabbits	↓	Palinski *et al.* 1995a
OxLDL/nLDL s.c.	α-oxLDL IgG ↑	NZ rabbits	↓	Ameli *et al.* 1996
Cholesterol-rich liposomes i.m.	α-cholesterol IgG, IgM↑	NZ rabbits	↓	Alving *et al.* 1996
MDA-LDL s.c.	α-MDA-LDL IgG ↑	apoE$^{-/-}$	↓	George *et al.* 1998b
MDA-LDL/nLDL i.p.	α-MDA-LDL IgG ↑/ ↔	LDLR$^{-/-}$	↓	Freigang *et al.* 1998
MDA-LDL s.c.	α-MDA-LDL IgG ↑	apoE$^{-/-}$	↓	Zhou *et al.* 2001
ApoB-100 peptides sequences	α-native and MDA-modified apoB-100 sequence IgG ↑	C57Bl/6	↓	Fredrikson *et al.* 2003b; Fredrikson *et al.* 2005
MDA-modified apoB-100 peptides sequences	α-MDA-modified apoB-100 sequences IgG ↑	apoE$^{-/-}$	↓	Fredrikson *et al.* 2005
ApoB-100 peptide sequence s.c./i.p.	α-apoB-100 sequence (peptide 2) IgM ↑	apoE$^{-/-}$	↓ (with peptide 2)	Chyu *et al.* 2005
Heat-killed R36a *Str. pneumoniae* s.c./i.p.	α-oxLDL IgM (T15) ↑, expansion of oxLDL-spesific B cells	LDLR$^{-/-}$	↓	Binder *et al.* 2003
Passive immunization with anti-MDA-modified apoB-100 IgG$_1$ i.p.		C57Bl/6	↓	Schiopu *et al.* 2004
Passive immunization with anti-MDA-modified apoB-100 IgG$_1$ i.p.		LDLR$^{-/-}$	↓	Ström *et al.* 2006
Passive immunization with anti-phosphocholine IgM i.p.		apoE$^{-/-}$	↓	Faria-Neto *et al.* 2005
Mycobacterial HSP65 i.c.	cellular immune response to HSP65 ↑	New Zealand rabbit	↑	Xu *et al.* 1992
Mycobacterium tuberculosis/ HSP65 s.c.	cellular immune response to HSP65 ↑	C57Bl/6	↑	George *et al.* 1999b
Mycobacterium tuberculosis/ HSP65 s.c.	cellular immune response to HSP65 ↑, α-HSP65 IgG ↑	LDLR$^{-/-}$	↑	Afek *et al.* 2000
HSP65 p.o.	cellular immune response to HSP65 ↓, Th2 type response ↑	LDLR$^{-/-}$	↓	Harats *et al.* 2002
HSP65 p.o./intranasally	cellular immune response to HSP65 ↓, Th2 type response ↑	LDLR$^{-/-}$	↓	Maron *et al.* 2002
β2GPI s.c.	both cellular and humoral immune response to β2GPI ↑	LDLR$^{-/-}$	↑	George *et al.* 1998a
β2GPI s.c.	humoral immune response to β2GPI ↑	apoE$^{-/-}$	↑	Afek *et al.* 1999
β2GPI p.o.	cellular immune response to β2GPI ↓	LDLR$^{-/-}$	↓	George *et al.* 2004
IL-12-PADRE vaccination i.m.	generation of antibodies that blocked IL-12 function	LDLR$^{-/-}$	↓	Hauer *et al.* 2005

s.c., subcutaneously; i.c., intracutaneously; i.p., intraperitoneally; p.o., perorally; i.m., intramuscularly

many pro-inflammatory effects would rather promote atherogenesis. Adaptive immunity as a whole has been suggested to mediate proatherogenic effects. Thus, mice that are deficient in Rag-1 or Rag-2, which leads to a lack of functional T and B lymphocytes, develop less atherosclerosis than control mice. However, this effect was observed only at lower serum cholesterol levels, and the effect disappeared when the mice were fed a high-fat diet and developed higher serum cholesterol levels (Dansky *et al.* 1997; Daugherty *et al.* 1997; Reardon *et al.* 2001; Song *et al.* 2001). At high cholesterol levels, the protective effect of immune deficiency may be overruled by excessive hypercholesterolemia or, alternatively, qualitative differences of immune activity, such as a shift from the Th1 to the Th2 type of response, may occur under severe hypercholesterolemia and hence explain the results. Consistently, disruption of costimulatory signaling (such as CD40-CD40L and B7-1/2-CD28), which is essential for the efficient activation of adaptive immunity, decreases atherosclerosis (Mach *et al.* 1998; Buono *et al.* 2004). Furthermore, intravenous injection of immunoglobulin G, which is known to modulate and suppress the immune response by various mechanisms, decreases atherosclerosis (Nicoletti *et al.* 1998). Interestingly, a recent study showed that this effect actually depended on the intactness of the complement system (Persson *et al.* 2005). Furthermore, it has been shown that pro-atherogenic effects of adaptive immunity are mediated mainly via CD4+ cells, since transfer of CD4+ cells from atherosclerotic mice to immunodeficient recipients aggravated atherosclerosis (Zhou *et al.* 2000), especially when the T cell population was derived from oxLDL-immunized mice (Zhou *et al.* 2006). However, the current concepts suggest that different subgroups of CD4+ cells may have opposite effects on atherosclerosis. Thus, while evidence indicates that the Th1 type of immune response is pro-atherogenic, the Th2 type of response may confer some atheroprotective effects. Furthermore, both types of responses are under control of regulatory T cells, which, by suppressing and controlling immune activation, mediate anti-atherogenic effects. In addition, B cells have been suggested to perform mainly atheroprotective functions. In support of this notion, complete B cell deficiency increased both the early and late stages of atherosclerosis in $LDLR^{-/-}$ mice (Major *et al.* 2002). In addition, removal of the spleen, which harbors a significant proportion of memory B cells and plays an important role in B cell maturation, significantly increased atherosclerosis in $apoE^{-/-}$ mice, which effect was reversed by the transfer of B cells from atherosclerotic $apoE^{-/-}$ mice (Caligiuri *et al.* 2002).

In addition to genetic modifications, various immunization studies have given insights into the role of immune response in atherogenesis (Tables 4 and 5). Since various studies have suggested that activation of the immune response might have harmful effects in atherogenesis, and elevated antibody levels against oxLDL correlated with the extent of atherosclerosis, it was somewhat surprising that parenteral immunization of animals with MDA-modified or Cu^{2+}-oxidized LDL or with peptide fragments of MDA-modified apoB-100 suppressed atherogenesis. In these studies, a decrease in the extent of atherosclerosis correlated with an increase in T cell-dependent IgG antibodies against immunogen, suggesting that antibodies might be involved in mediating this protective effect. In addition, immunization seemed to promote the Th2 type of response, which was also involved in the induction of natural IgM antibodies that have been suggested to exert atheroprotective effects (Fredrikson *et al.* 2005; Binder *et al.* 2004; Binder *et al.* 2003). Similarly, passive immunization of $apoE^{-/-}$ mice with IgG_1 antibodies against MDA-modified apoB-100 sequences inhibited atherosclerosis (Schiopu *et al.* 2004). In contrast to oxLDL, parenteral immunization with HSP65 has been shown to aggravate atherosclerosis in animal models. In immunized animals, the T cell proliferative response to HSP65 was increased, whereas the production of anti-HSP antibodies did not correlate with the extent of lesions, suggesting that activation of cell-mediated immunity might be responsible for the proatherogenic effects of HSP immunization,

possibly via an autoimmune attack against stressed vascular cells. Since mucosal administration of antigens has been shown to induce tolerance by a mechanism probably involving regulatory T cells (Cottrez & Groux 2004), Maron *et al.* tested whether mucosal administration of HSP65 would affect the extent of atherosclerosis. Indeed, in response to oral/nasal administration of HSP65, atherosclerosis-prone mice developed less atherosclerosis, and the effect was associated with suppressed T cell reactivity against HSPs, decreased IFNγ-production, and increased expression of Th2-dependent antibodies and cytokines (Maron *et al.* 2002; Harats *et al.* 2002). Similarly, although parenteral immunization with β2GPI aggravated atherosclerosis in both $LDLR^{-/-}$ and $apoE^{-/-}$ mice (George *et al.* 1998a; Afek *et al.* 1999), induction of oral tolerance significantly decreased atherosclerosis (George *et al.* 2004). Thus, it appears that regulatory T cells play an important role in mediating atheroprotective effects and might hence present a possible target for therapeutic developments. The type of immune responses evoked by different immunization routes and their effects on atherogenesis are further summarized in Table 5.

Table 5. Summary of the effects of immunization or induction of tolerance on atherosclerosis in mouse models

	Immunological response	**Atherosclerosis**
Parenteral immunization		
oxLDL/MDA-LDL/apoB peptides	IgG and IgM titers against antigen ↑ Th2 type of immune response ↑	↓
HSP65	Proliferative response of T cells to HSP ↑	↑
β2GBI	Ig titers against β2GBI ↑ Proliferative response of T cells to β2GBI ↑	↑
Heat-killed R36a *Str. pneumoniae*	IgM titers against oxLDL phosphocholine ↑ Expansion of oxLDL-spesific T15-IgM-secreting B cells	↓
Mucosal immunization - tolerance		
HSP65	Proliferative response of T cells to HSP ↓ IgG titers against HSP ↓ Expression of Th1 cytokines ↓ Expression of Th2 cytokines ↑	↓
β2GBI	Proliferative response of T cells to β2GBI ↓ Th2 cytokines ↑ Th1 cytokines ↔	↓
Neonatal immunization - tolerance		
oxLDL	IgG and IgM titers ↓ proliferative response of T cells to oxLDL ↓	↓
Passive immunization with antigen-specific antibodies		
Anti-MDA-apoB peptides (IgG_1)		↓
Anti-phosphocoline (IgM)		↓

III AIMS OF THE STUDY

Complement system is part of the innate immune system. Studies on experimental animals suggest that complement system also plays a role in atherogenesis. Complement is activated in atherosclerotic lesions, and lesions have been shown to contain various activators of the complement system, e.g. modified lipoproteins and oxLDL-IgG immune complexes. When activated, the cascade-like activity of the complement generates various effector molecules (opsonins, anaphylatoxins, C5b-9), which can regulate the inflammatory response and act together to destroy their target structures. To prevent uncontrolled complement activation or its attack against normal host cells, complement needs to be under strict control by regulatory proteins.

The present thesis concentrates on elucidating the role of the complement regulators, effectors and activators in atherosclerotic lesions. The specific aims of the study were:

1. To analyze the presence and localization of two complement regulators, factor H and C4bp, in human atherosclerotic lesions, and to elucidate their functional role in atherosclerotic lesions (Studies I and II).

2. To analyze whether receptors of the two complement-derived anaphylatoxins, C3a and C5a, are present in atherosclerotic lesions, and to characterize the cell types expressing them (Study III).

3. To investigate, whether oxLDL-IgG immune complexes, in addition to activating the classical complement pathway, could have direct effects on the intimal cells. Spesifically, we wanted to study, whether oxLDL-IgG immune complexes affect the survival of cultured human monocytes and what are the receptors and downstream signaling events involved in this process (Study IV).

IV MATERIALS AND METHODS

The methods used in this study are summarized in Table 6, and the techniques have been described in greater detail in the original publications listed in the Table. Thus, only a short overview of the materials and methods used is provided here. The antibodies used in the immunohistochemistry, Western blotting, and blocking experiments are listed in Table 7. In the body of the text, no references are given, and they can be found in the respective publications.

Table 6. Methods

Method	Used in publications	Reference
Immunohistochemistry	I, II, III	
Extraction and purification of proteoglycans	I, II	Öörni *et al.* 1997
Affinity chromatography	I, II	
Surface-plasmon resonance analysis	I	Jokiranta *et al.* 2001
Complement activation assays	I	
Western blotting	I, IV	
RNA isolation	III	
RT-PCR	III	
Isolation of LDL	I, IV	Havel *et al.* 1955
Modifications of LDL		
oxidation	IV	
enzymatic modification	I	Chao *et al.* 1992
Determination of TBARS	IV	Hessler *et al.* 1983
High-performance thin-layer chromatography	IV	
Isolation and culture of of human monocytes	III, IV	Saren *et al.* 1996
Determination of endotoxins	IV	
Protein quantitation with Lowry	IV	Lowry *et al.* 1951
Preparation of immune complexes	IV	
Isolation of human anti-oxLDL antibodies	IV	Koskinen *et al.* 1998
Determination of M-CSF with ELISA kit	IV	
Cell viability assay (WST)	IV	
Thymidine incorporation assay	IV	
Determination of apoptosis:		
TUNEL	IV	
DNA fragmentation (ELISA)	IV	
active caspase-3 (ELISA)	IV	
Blocking studies:		
blocking the effect of M-CSF	IV	
blocking the activation of the Akt pathway	IV	
blocking Fcγ receptors	IV	

Human coronary artery and aortic samples

Coronary artery specimens and aortas were obtained at autopsy at the Department of Forensic Medicine, University of Helsinki, 27 to 216 hours after the death of patients who died of non-cardiac causes. Segments with and without evident atherosclerotic plaques were taken from the left anterior descending arteries. In addition, segments of the left and right human coronary arteries were obtained freshly from recipient hearts at the time of cardiac

transplantation or from hearts of organ donors who had been excluded from transplantation due to size, tissue type mismatch, or coronary atherosclerosis in Helsinki University Central Hospital. Informed consent was obtained from each patient. Patients with malignancies or chronic inflammatory diseases were excluded from the study. Institutional Ethics Committees had approved the study protocol, and the study was conducted in accordance with the Helsinki Declaration.

The samples were either embedded in OCT and snap-frozen in liquid nitrogen, or fixed in 10% neutral-buffered formalin and embedded in paraffin according to standard procedures. Frozen (8 μm) or paraffin (4 μm) sections were cut, stained with hematoxylin-eosin, and evaluated microscopically to classify the atherosclerotic lesions according to the guidelines of the American Heart Association. For PCR analysis, only freshly obtained coronary samples were used to avoid any bias caused by the postmortem changes in RNA. These segments were snap-frozen in liquid nitrogen, after which the adventitia of the coronary arteries was carefully removed and the absence of the adventitial layer was verified by Masson Trichrome staining. The division of samples into normal and atherosclerotic ones was done based on macroscopic analysis.

Extraction and purification of aortic proteoglycans

Proteoglycans were isolated from the intima-media of human aortas obtained at autopsy within 24 h of accidental death. Briefly, proteoglycans were extracted from intima-media at 4°C for 24 h with 15 volumes of buffer containing 6 M urea and protease inhibitors. After extraction, the mixture was centrifuged at 100 000 g for 60 minutes. The supernatant was diluted with 6 M urea to give a final concentration of 0.25 M NaCl and loaded on a HiTrap Q column. The proteoglycans were eluted with a linear gradient of 0.25 to 1.0 M NaCl, and the peaks at 280 nm were collected, dialyzed against water and lyophilized. The quantities of proteoglycans were expressed in terms of their glycosaminoglycan contents.

Isolation and modifications of low-density lipoprotein

Human LDL (d = 1.019-1.050 g/ml) was isolated from plasma of healthy volunteers by sequential ultracentrifugation under aseptic conditions in the presence of 3 mM EDTA. LDL was dialyzed to a buffer containing 1 mM EDTA and 150 mM NaCl (pH 7.4).

E-LDL was prepared by modification of LDL by trypsin and cholesterol esterase. Briefly, LDL (2 mg/ml protein) was first treated with trypsin from bovine pancreas in PBS at +37°C for 2 h after which trypsin was inhibited with 64 μg/ml Soybean trypsin inhibitor in PBS. Finally the mixture was incubated with 80 μg/ml cholesterol esterase from *Candida cylindracea* in PBS at +37°C for 2 h. E-LDL was purified by gel filtration on a Bio-Gel A-0.5m column, and its concentration was determined from its cholesterol content.

Oxidized LDL was prepared by first removing EDTA from LDL by PD-10 column, and then incubating LDL (1 mg/ml) with 10 μM $CuSO_4$ at 37°C for 18 h. Oxidation was terminated by the addition of EDTA (100 μM) and by cooling the sample on ice. The extent of oxidation was determined by measuring thiobarbituric acid-reactive substances (TBARS).

Isolation and culture of human monocytes

Human mononuclear leukocytes from healthy subjects were isolated from buffy by Ficoll-Paque gradient centrifugation. Mononuclear cells were resuspended in DMEM supplemented with penicillin and streptomycin, and plated on cell culture wells. After 30 min at 37°C in 5% CO_2, the cells were washed with PBS to remove non-adherent cells, and SFM medium supplemented with antibiotics was added. After 18 h, the cells were washed three times with PBS, and experiments with monocytes were started with fresh SFM medium supplemented with antibiotics. For some experiments, monocytes were cultured in the presence of GM-CSF (10 ng/ml) for 7 days to allow their differentiation into macrophages.

Cells of the human monocytic cell-line THP-1 were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. To induce their maturation, the cells were grown in the presence of 200 nM phorbol 12-myristate 13-acetate (PMA) for 3 days.

Isolation of anti-oxLDL antibodies

Anti-human oxLDL antibodies were isolated from freshly obtained human serum. Briefly, LDL was coupled to a NHS-HiTrap column and oxidized by 10 μM $CuCl_2$ for 18 h at +37°C. Oxidation was finished by extensive washing with buffer containing 200 μM butylated hydroxytoluene and 300 μM EDTA. To isolate anti-oxLDL antibodies, 10 ml of serum, obtained from healthy volunteers, was diluted 1:4 in 0.01 M $NaHCO_3$, pH 8.3 and applied into the oxLDL column. After 18 hours' incubation at +4°C, the column was extensively washed with the buffer, after which anti-oxLDL antibodies were eluted from the column with 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3. The protein concentrations of eluate fractions were determined by the method of Lowry. The purity of eluted antibodies was verified with SDS-PAGE, and ELISA assay was done to confirm that the isolated antibodies specifically bound to oxLDL.

Human recombinant antibodies (clones LDO 107 Z3 IEI-E3 and CT-17, both IgG_1) against MDA-modified apoB-100-derived peptides were a kind kift from Dr. B. Jansson (BioInvent International AB, Lund, Sweden).

Immunohistochemistry

Frozen and paraffin sections were stained by using an indirect immunoperoxidase or immunofluoresence method. Briefly, the paraffin sections were deparaffinized in xylene and rehydrated through a series of graded alcohols. The frozen sections were fixed either in methanol for 10 minutes or in ice-cold acetone for 2 minutes. The slides were incubated with 2% H_2O_2 to block endogenous peroxidase activity and then with 3% normal serum (rabbit, horse, or goat) to reduce non-specific binding of antibodies, each for 30 minutes at room temperature. Primary antibodies were incubated overnight at +4°C. To obtain a color signal, the primary antibodies were detected using the commercial avidin-biotin complex system with 3-amino-9-ethylcarbazole as a chromogen. Thereafter, the sections were counterstained with hematoxylin and mounted using Aquamount or Permount. To obtain a fluorescent signal, the primary antibodies were detected using various isotype-specific Alexa-conjugated secondary antibodies, or alternatively, the biotinylated secondary antibodies were detected by incubating the slides with streptavidin-FITC. The nuclei were stained with 4',6-diamino-2-phenylindole (DAPI), and the sections were mounted using fluorescent mounting media. As negative controls, the primary antibodies were replaced with non-specific rabbit IgG or isotype-matched mouse IgGs.

Western blotting

Immunoblotting was used to analyze the proteins of homogenates of human arteries and lysates of human monocytes. In brief, samples were run into a 10% SDS-page gel under nonreducing conditions and electrotransferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked by incubating the membrane for 1 hour in 5% fat-free milk. The membranes were then probed overnight at +4°C with primary antibodies (see table 7). After washing, the membranes were incubated for 1 h at room temperature with HRP-conjugated secondary antibodies and washed, and the immunoreactive bands were visualized on an x-ray film by enhanced chemiluminescence. In some experiments, the membranes were then stripped and probed again overnight at +4°C with other primary antibodies. The immunoblots were quantitated by densitometric scanning with a Gel Doc 2000 gel documentation system.

Affinity chromatography and surface-plasmon resonance analysis

Human aortic proteoglycans were coupled to a NHS-activated HiTrap column, and human factor H, C4bp, or CRP was injected into the column that had been equilibrated with buffer containing 10 mM HEPES, pH 7.4, and either 2 mM $CaCl_2$ or 100 µM EDTA. The material bound to the column was then eluted with a gradient of NaCl (0 → 250 mM) in a high-performance FPLC system. The elution was monitored by UV absorbance at 280 nm and the NaCl gradient by conductometry.

Surface plasmon resonance measurements were performed using the BIACORE 2000® instrument and the BIAevaluation software V 3.0 as described previously (Jokiranta *et al.* 2001). Human factor H and human CRP were immobilized on carboxylated dextran CM5 sensor chips, and analyses of binding with human aortic proteoglycans were performed using 1/3 veronal-buffered saline. In control experiments, the sensor chips were treated identically, except that no protein was coupled to the chip.

RNA isolation and RT-PCR

For PCR analysis, only freshly obtained coronary samples were used in order to avoid any bias caused by postmortem changes in RNA. Total RNA was isolated from the intima-medias of coronary arteries using ultra-pure TRIzol reagent and a commercial RNA isolation kit, including deoxyribonuclease digestion. After that, 0.25 µg of purified total RNA was transcribed into cDNA with a Superscript TM pre-amplification system. The PCR product was verified by DNA sequencing to represent the corresponding target. The RT-PCR assay was standardized to the expression level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Preparation of insoluble oxLDL-IgG immune complexes

Before preparing oxLDL-IgG immune complexes, rabbit anti-LDL antibody was dialyzed against sterile PBS to remove NaN_3. Insoluble ICs were prepared by incubating rabbit anti-LDL antibody (200 µg/ml) with oxLDL (125 µg/ml) or in sterile PBS overnight at +4°C. The concentrations of oxLDL and anti-LDL antibodies used to prepare ICs were determined by a precipitin curve. After incubation, the precipitate was centrifuged at 10 000 rpm and resuspended in sterile PBS. The protein content of oxLDL-IgG ICs was determined by the Lowry assay and the cholesterol content with a commercially available kit. In some

Table 7. List of primary antibodies

Antigen	Clone/cat.no.	Host	Isotype	Working dilution	Source/ref.	Study
Immunohistochemistry						
C3d	A0063	rabbit		1:500	DAKO	I, III
sC5b-9 neoantigen	A239	mouse	IgG_{2bk}	3.3 µg/ml	Quidel	I, II, III
ApoB-100	2-B4	mouse	IgG_{2b}	10 µg/ml	ICN Pharm	I
CRP	CRP-8	mouse	IgG_1	29 µg/ml	Sigma	I
CRP	A29	rabbit		1:500	Biomeda	I
Versican LF99		rabbit		1:100	Bernstein *et al.* 1995	I
Factor H		goat		1:300	Incstar	I
C3aR	8H1	mouse	IgG_1	10 µg/ml	BD	III
C3aR	BIIG1	mouse	IgG_{2a}	8 µg/ml	Monsinjon *et al.* 2001	III
C3aR	BIIG2	mouse	IgG_{2a}	8 µg/ml	Monsinjon *et al.* 2001	III
C3aR		rabbit		4 µg/ml	Gasque *et al.* 1998	III
C3aR	sc-14621	goat		1 µg/ml	SCBT	III
C3aR	ab7209	rabbit		10 µg/ml	Abcam	III
CD88	S5/1	mouse	IgG_{2a}	10 µg/ml	Serotec	III
CD88	W17/1	mouse	IgG_1	10 µg/ml	Serotec	III
Macrophage	HAM56	mouse	IgG_1	0.7µg/ml	DAKO	I, III
CD68 (macrophage)	PG-M1	mouse	IgG_3	7.2 µg/ml	DAKO	I, III
CD43 (T cell)	DF-T1	mouse	IgG_1	2.65 µg/ml	DAKO	III
CD45RO	UCHL-1	mouse	IgG_{2a}	7.5 µg/ml	DAKO	III
CD3 (T cell)	A0452	rabbit		10 µg/ml	DAKO	III
Tryptase (mast cell)	G3	mouse	IgG_1	1.5 µg/ml	DAKO	III
Tryptase (mast cell)		rabbit		0.6 µg/ml	Harvima *et al.* 1988	III
Actin (muscle)	HHF35	mouse	IgG_1	1 µg/ml	DAKO	III
α-actin (SMC)	1A4	mouse	IgG_{2a}	0.5 µg/ml	DAKO	III
CD31 (endothelial cell)	JC/70A	mouse	IgG_1	10 µg/ml	DAKO	III
VE-cadherin	BV6	mouse	IgG_{2a}	10 µg/ml	Chemicon	III
HLA-DP, DQ, DD antigen	CR3/43	mouse	IgG_1	2.3 µg/ml	DAKO	III
C4bp		mouse	IgG	5.5 µg/ml	Hardig *et al.* 1997	II
C4bp	A215	mouse	IgG_{2bk}	20 µg/ml	Quidel	II
Protein S	HS3	mouse	IgG_1	20 µg/ml	Sigma	II
Cleaved caspase-3	9661	rabbit		1:50	Cell Signaling	II
Properdin		goat		1.6 µg/ml	Incstar	II
Western blotting						
Factor H		goat		1:5000	Calbiochem	I
SCR19-20		rabbit		1:1000	A kind gift from Dr. Jens Hellwage	I
Factor H 196X8		mouse	IgG	5 µg/ml	Jokiranta *et al.* 1996	I
Phospho-Akt	4058S	rabbit (mAb)		1:1000	Cell signaling Tech.	IV
Akt	c-20	goat		0.4 µg/ml	Santa Cruz	IV
Blocking experiments						
M-CSF	26730	mouse	IgG_{2a}	10 µg/ml	R&D Systems	IV
CD64 (FcγR I)	10.1	mouse	$F(ab)_2$	20 µg/ml	Ancell	IV
CD32 (FcγR II)	7.3	mouse	$F(ab)_2$	20 µg/ml	Ancell	IV
Preparation of ICs						
LDL	BT-905	rabbit			Biomedical Technologies	IV
KLH	H0892	rabbit			Sigma	IV

experiments, native LDL-IgG ICs and keyhole limpet hemocyanin (KLH) -IgG ICs were prepared by replacing oxLDL with nLDL or incubating rabbit anti-KLH antibody together with KLH, respectively. To prepare insoluble immune complexes containing human IgG, soluble immune complexes between oxLDL and human anti-oxLDL antibodies (either recombinant or isolated from serum) were further linked by the $F(ab)_2$ fragment of goat anti-human IgG. The endotoxin levels of oxLDL-IgG ICs and other proteins used in the experiments were analyzed by Limulus assay and found to contain endotoxin levels lower than 100 pg/ml of LPS.

Determination of cell viability, proliferation, and apoptosis

To analyze the viability of the cells, 50 µl of Cell Proliferation Reagent WST-1 was added to the cells, which were incubated for 1 h at +37°C in 5% CO_2, and the production of formazan dye was quantified by measuring absorbance at 450 nm. To analyze cell proliferation, monocytes were cultured in the presence of [^{3}H-methyl]-thymidine and then harvested on a TomTek harvester. Incorporated [^{3}H-methyl]-thymidine was measured with a liquid scintillation counter. In the experiments designed to study apoptosis, the monocytes detached to the media were collected by centrifugation and analyzed together with the cells attached to the wells. Nuclear fragmentation and levels of active caspase-3 were analyzed by commercial ELISA-based methods. For annexin V staining, monocytes were cultured on glass slips, fixed with 1% paraformaldehyde, and incubated with Annexin V Fluos diluted in HEPES buffer.

V RESULTS AND DISCUSSION

1 Complement activation in atherosclerosis

1.1 Regulation of complement activation in atherosclerotic lesions (I and II)

1.1.1 Factor H and C4bp are present in atherosclerotic lesions

In an initial attempt to better understand the regulation of the complement system in atherosclerotic lesions, we analyzed the presence of two complement regulators, factor H and C4b-binding protein (C4bp), in different stages of atherosclerosis progression by immunohistochemical methods and compared their staining patterns with those of the activators of the complement system, the activation or inactivation products of the complement as well as the terminal membrane-attack complex (C5b-9).

We found that factor H, the major inhibitor of the alternative pathway, was already present in normal intimas as a narrow subendothelial layer (Figure I, online supplement, in paper I). In contrast, positive staining was more extensive in atherosclerotic lesions. Thus, in early to intermediate lesions, factor H was present in the superficial intima, in which it showed strict colocalization with versican proteoglycan (Figure II, online supplement, and Figure 1, in paper I). This colocalization was observed in all early atherosclerotic lesions in which a distinct superficial proteoglycan-rich layer and a deep musculoelastic layer could be clearly distinguished. In contrast, in advanced atherosclerotic lesions, the staining for factor H was more extensive and diffuse, and showed only partial colocalization with versican proteoglycan. In all lesions, however, the deep intima did not stain for factor H. In addition to immunohistochemistry, we verified the presence of factor H and also other members of the factor H family by immunoblotting homogenates of human coronary arteries and aorta (Figure 5, in paper I). These analyses revealed that factor H and factor H-like protein-1 (FHL-1), which is formed by an alternatively spliced mRNA of the factor H gene and shares the complement-regulatory properties of factor H, showed preferential retention in the intima when compared to their levels in plasma. Indeed, although it is likely that part of the intimal factor H derives from plasma through the endothelial layer, the increased levels of factor H may also result from the local synthesis of factor H by intimal cells, since at least macrophages and endothelial cells have been shown to be able to synthesize this complement inhibitor (Friese *et al.* 1999; Brooimans *et al.* 1989). Although factor H has been previously demonstrated in carotid lesions (Seifert & Hansson 1989), this was the first study to demonstrate factor H in human coronary arteries and show its colocalization with proteoglycans.

Similarly to factor H, we found that C4bp, the major inhibitor of the classical complement pathway, was present both in normal intima and in atherosclerotic lesions of human coronary arteries. In the normal arterial intima, oonly the very subendothelial intima lining the vessel lumen showed a positive signal for C4bp, whereas in early to intermediate lesions, C4bp was clearly visible in the whole superficial layer of the intima with no positive staining in the deepest parts of the intima. In advanced lesions, C4pb showed a more diffuse and extensive staining pattern, but the deepest part of the musculoelastic layer again lacked staining for C4bp (Figure 1, in paper II). Furthermore, in the superficial area, C4bp colocalized with glycosaminoglycans, components of arterial proteoglycans (Figure 2A, in paper II). While the staining for C4bp was mostly extracellular, some cells in the superficial intima and adventitia

also showed positive staining for C4bp. These findings are consistent with some previous studies, which have shown that C4bp is present in atherosclerotic lesions of the human thoracic aorta (Kimoto *et al.* 1996), and which have also revealed mRNA for C4bp in arterial wall (Yasojima *et al.* 2001a). However, our study illustrates, for the first time, the presence of C4bp in the intima during different stages of coronary atherosclerosis and demonstrates its colocalization with arterial proteoglycans.

In our study, in addition to learning about the lesional distribution of C4bp, we were also interested in whether C4bp would colocalize with protein S in the arterial wall. Protein S is a vitamin K-dependent cofactor in the anticoagulant system, and the majority of C4bp circulates as a complex with protein S (reviewed by Villoutreix *et al.* 1999). Double-immunofluorescence staining revealed that C4bp and protein S colocalized in the extracellular space of the superficial intima, in addition to which similar colocalization was also observed in some subendothelial cells (Figure 5, in paper II). In addition, an intense cellular staining pattern for protein S was detected in the endothelial lining of the vessel wall, in majority of intimal cells, and especially in the media, which findings are consistent with the previous reports showing that protein S binds to endothelial cells (Hackeng *et al.* 1993) as well as with the reports on the synthesis of this protein by endothelial and vascular smooth muscle cells *in vitro* (Fair *et al.* 1986; Benzakour & Kanthou 2000). Of interest, the interaction of C4bp and protein S may also have some functional importance. Thus, upon the formation of this complex in the circulation, the anticoagulant activity of protein S is lost, whereas the functional properties of C4bp are not affected. In tissues, protein S has been suggested to bind to apoptotic cells and to increase their uptake by phagocytes. When present as a complex, protein S can also carry C4bp to the surface of apoptotic cells, which markedly decreases the uptake of apoptotic cells by phagocytes (Webb *et al.* 2002; Kask *et al.* 2004). However, the targeting of C4bp on the surface of apoptotic cells could still be beneficial, since it can prevent further complement activation, which would lead to secondary necrosis and increased inflammation. Interestingly, in our study, we could observe protein S colocalized with cleaved caspase-3-positive apoptotic cells. In addition, some apoptotic cells were positive also for C4bp (Figure 6, in paper II). These results suggest that protein S and C4bp could be involved in the regulation of the disposal of apoptotic cells and in diminishing the inflammatory response even in atherosclerotic lesions.

The aim of this part of our study was to find out whether complement activation is regulated in atherosclerotic lesions. Regarding the inhibition of complement activation, factor H and C4bp play analogous roles in the alternative and classical pathways, respectively (see Figure 3). More specifically, factor H binds C3b and thus inhibits the assembly and promotes the decay of alternative pathway C3 convertase and, in addition, acts as a cofactor for factor I in the proteolytic cleavage of C3b (Whaley & Ruddy 1976; Weiler *et al.* 1976; Pangburn *et al.* 1977). By these mechanisms, factor H acts at the most critical step of the complement activation, the amplification loop, and is thus largely responsible for the prevention of uncontrolled complement activation. C4bp, in turn, inhibits the classical complement pathway by preventing the assembly and accelerating the decay of the classical pathway C3 convertase C4b2b and acting as a cofactor for factor I in the cleavage and inactivation of C4b (Gigli *et al.* 1979). Thus, inhibition of complement activation via factor H and C4bp prevents the activation of the terminal complement pathway, but allows the generation of complement-derived opsonins, i.e. the split products of C3 and C4.

We initially observed that, in atherosclerotic coronary lesions, C4c/d and C3d, two products of complement activation by the classical and alternative pathways, respectively, did not

necessarily colocalize with the terminal product of complement activation, C5b-9 (Figure 1, in paper I; Figure 4, in paper II). More specifically, in early to intermediate lesions, C4c/d and C3d were present in the superficial proteoglycan-rich layer of the intima, whereas C5b-9 was found as distinct deposits deeper in the intimal musculoelastic layer with very little or no overlap with C4c/d or C3d. This finding suggested to us that, in the superficial intima, complement activation had been prevented from proceeding to the terminal C5b-9 level. Interestingly, as described above, both C4bp and factor H located specifically in this superficial area devoid of C5b-9, suggesting that these two regulators could have inhibited complement activation in the area. To obtain further evidence to support this hypothesis, we analyzed in more detail the spatial relationship between factor H or C4bp and C5b-9 by immunofluorescence double staining of sections of coronary arteries (Figure 2, in paper I; Figure 3, in paper II). Indeed, we found a reciprocal staining pattern for factor H or C4bp and C5b-9 in most of the samples studied. To obtain semiquantitative data, we adopted a scoring system for the analysis of the proportion of overlap between the areas positive for C5b-9 and factor H. We found that, in the samples with distinct stainings for factor H and C5b-9, about 60% of the lesions showed almost fully reciprocal staining patterns (≤ 20% overlap), while 25% showed some overlap (21-80% overlap), and only 12.5% showed colocalization between factor H and C5b-9 (>80% overlap). Similarly, overlap between C4bp and C5b-9 was very scarce, and in many cases their localization patterns were mutually exclusive. These novel findings suggested to us that complement activation in the arterial wall was regulated, and that the two soluble inhibitors, C4bp and factor H, could participate in this regulatory process.

Taken together, our results suggested that both the classical and alternative pathways of complement were activated in atherosclerotic lesions. However, in the superficial intima, complement activation is limited to the C3 level and does not proceed to the assembly of terminal C5b-9. Furthermore, the superficial proteoglycan-rich layer of the intima contained inhibitors of the alternative and classical pathways, factor H and C4bp, respectively, suggesting that they could have inhibited complement activation in this area. To support this idea, these regulators showed a strictly reciprocal staining pattern with the complement terminal product C5b-9, which was found deeper in the musculoelastic layer of the intima. In addition, we could also show that CRP, which has the ability to initiate the classical complement pathway but also to terminate complement activation at the C3 level by binding factor H (Jarva *et al.* 1999), was present in the superficial intima, thus further supporting a role for factor H in the regulation of the complement in this layer of the human coronary arterial wall.

1.1.2 Factor H and C4bp bind to arterial proteoglycans

Since immunohistochemical analysis demonstrated that factor H and C4bp were located in the superficial intima and colocalized with arterial proteoglycans, we also tested for their ability to bind to arterial proteoglycans *in vitro*. Proteoglycans were isolated from human aorta and coupled to the NHS-activated HiTrap column, and the binding of factor H and C4bp to the column was analyzed by affinity chromatography (Figure 3B, in paper I; Figure 2B, in paper II). In addition, the surface plasmon resonance technique was used to analyze the binding of factor H (Figure 3A, in paper I). These analyses revealed that both factor H and C4bp bound to arterial proteoglycans. Addition of EDTA did not affect the binding, showing that these interactions were independent of the presence of Ca^{2+}. Even though we could also detect CRP in the proteoglycan-rich layer of the intima, it did not bind to arterial proteoglycans *in vitro* (Figure III, online supplement, in paper I).

Previously, factor H has been shown to have three heparin-binding sites, one in each of its short consensus repeats 7, 9, and 20 (Pangburn *et al.* 1991; Blackmore *et al.* 1996; Blackmore *et al.* 1998; Ormsby *et al.* 2005), and it is thus likely that factor H binds to the glycosaminoglycan side chains of proteoglycans via one or more of these sites. Although the direct binding of factor H to arterial proteoglycans in affinity chromatography was relatively weak, it might still be physiologically significant because of the potentially multiple interactions between polymeric proteoglycans and factor H, and because the binding of factor H usually occurs after initial deposition of C3b, which can interact with factor H with three distinct sites (Cheng *et al.* 2006). Interestingly, the binding of heparin to factor H has been shown to potentiate the complement-inhibitory effect of factor H in the inactivation of C3b (Boackle *et al.* 1983).

Similar to factor H, C4bp has also been shown to contain heparin-binding sites, which are located in the short consensus repeat 2 of each of the seven α-chains (reviewed by Blom 2002). However, in contrast to factor H, the binding of soluble heparin glycosaminoglycan to C4bp actually inhibits its complement-regulatory properties. This is due to the fact that soluble heparin, by binding to each of the 7 heparin-binding sites in C4bp, prevents mutual binding of C4b and thereby inhibits the complement-regulatory function of C4b (Hessing *et al.* 1990). In contrast, the occupation of a restricted number of heparin-binding sites upon the binding of C4bp to immobilized glycosaminoglycans, like those in the arterial proteoglycan matrix, is the likely explanation for the ability of C4bp to exert its complement-regulatory function, as shown in our study. Similarly, various bacteria have been shown to bind C4bp to the heparin-like structures on their surface and in that way to protect themselves from complement-mediated killing by the host (reviewed by Blom *et al.* 2004).

1.1.3 Arterial proteoglycans inhibit complement activation

Since immunohistochemical analysis suggested that the proteoglycan-rich layer of the arterial wall is protected from the activation of the terminal complement pathway, and our *in vitro* experiments revealed that complement regulators bound to arterial proteoglycans, we hypothesized that arterial proteoglycans could be involved in the control of complement activation in atherosclerotic lesions. To study the role of proteoglycans in complement regulation, we undertook *in vitro* complement activation studies (Figure 4, in paper I). In these experiments we could show that, when coupled to Sepharose, both heparin and arterial proteoglycans were able to inhibit Sepharose-induced complement activation in human serum. By using another approach, we could show that heparin also inhibited complement activation induced by enzymatically modified LDL (E-LDL). Our findings are consistent with the previous reports, which have shown that heparin can inhibit complement activation (Kazatchkine *et al.* 1979), and they also add to the previous reports by showing similar effects for human arterial proteoglycans. Interestingly, the previous reports have shown that the inhibitory effect of heparin on complement activation appears to depend on its ability to bind to and potentiate the effect of factor H in the inactivation of C3b (Boackle *et al.* 1983).

Only a few previous studies have linked the extracellular matrix of the arterial intima to the regulation of complement activation in the intima. Niculescu *et al.* examined the relationship between C5b-9 and the complement inhibitor S-protein/vitronectin in atherosclerotic lesions (Niculescu *et al.* 1989). S-protein/vitronectin prevents C5b-9 insertion in the cell membrane by binding to C5b-7, which is then followed by generation of cytolytically inactive sC5b-9 complex. Using immuno-electron microscopy, they showed that S-protein/vitronectin appeared to associate with the connective tissue matrix, especially elastin fibers and collagen

bundles, and that cell debris in the vicinity of elastin also contained intense deposits of S-protein/vitronectin. In addition, all cell debris was positive for C5b-9, suggesting that at least some of it colocalized with S-protein/vitronectin. Thus, the results suggested that, by associating with S-protein/vitronectin, the connective tissue matrix plays a role in the regulation of the terminal complement pathway in atherosclerotic lesions. In addition, Hindmarsh *et al.* showed that the subendothelial extracellular matrix produced by human endothelial cells *in vitro* is protected from complement activation by strongly bound decay-accelerating factor (DAF), an inhibitor of both classical and alternative pathway C3 convertase (Hindmarsh & Marks 1998).

In summary, our findings suggested that the proteoglycan-rich layer of the arterial wall contains matrix-bound complement inhibitors, inhibits complement activation *in vitro*, and forms a protective area in which complement activation is restricted to the C3 level (Fig. 5). These results are novel and implicate that not only complement activation, but also inhibition, may play a role in atherogenesis. The exact effect of such inhibition of the complement system on the development of atherosclerosis is not fully understood. Based on the current knowledge, however, it can be hypothesized that inhibition of the complement system before

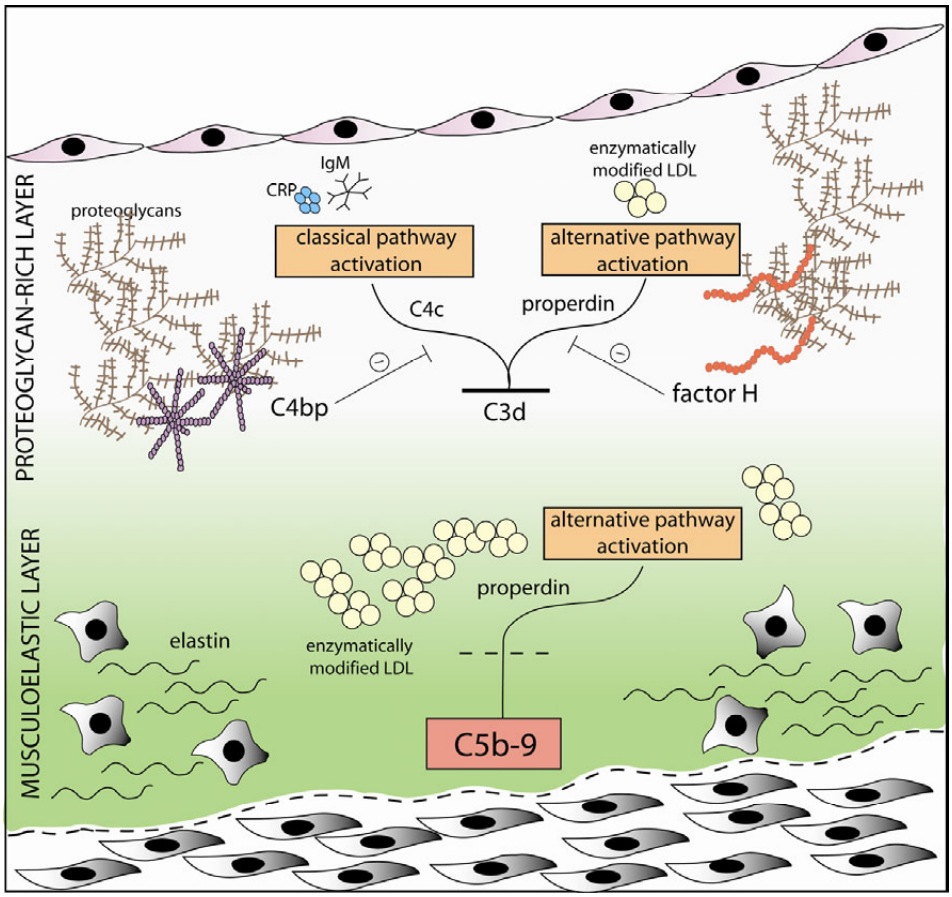

Fig. 5. Regulation of the complement system in atherosclerotic lesions. Our results suggested that, in the superficial proteoglycan-rich layer, which contained factor H and C4bp, complement activation was limited to the C3 level. Thus, although this area contained both activators of the complement system (IgM, CRP) and markers of activation via the classical (C4c) and alternative pathways (properdin), no terminal C5b-9 was present in the area. In the musculoelastic layer, possibly due to the absence of proper regulation, complement activation had proceeded to the assembly of C5b-9. Our results, which demonstrated the presence of properdin, but not C4c, in this area, suggested that C5b-9 had been generated as a result of activation of the alternative pathway of the complement. Previously, this area has been shown to contain modified lipids, which are known to activate complement via the alternative pathway.

the terminal level could be beneficial in atherogenesis by inhibiting the generation of pro-inflammatory C5a and C5b-9, but still allowing the generation of the opsonins C4b, C3b, iC3b, and C3d. Unfortunately, no experimental animal studies investigating the role of complement regulators in atherosclerosis exist. In humans, deficiencies of complement regulators are relatively uncommon. Genetic deficiency of factor H (about 20 cases described) leads to strongly decreased factor H levels in plasma, predisposes to bacterial infections, and causes haemolytic uremic syndrome (Thompson & Winterborn 1981) and glomerulonephritis (Levy *et al.* 1986). In patients with glomerulonephritis, the glomerular basement membranes, normally protected by extracellular matrix-bound factor H contain intense deposits of complement. Thus, our findings extend these findings and suggest that factor H also protects the proteoglycan-rich layer of the arterial intima from the complement attack. Unfortunately, no data are available regarding cardiovascular diseases in the small number of factor H-deficient patients. However, recent studies have investigated whether Y402H gene polymorphism of factor H, which locates within the binding site for heparin and C-reactive protein, plays a role in thromboembolic events. In two case-control studies, no evidence of an association between this polymorphism and the risk of incident of MI and ischemic stroke (Zee *et al.* 2005) or angiographically-verified coronary artery disease (Goverdhan *et al.* 2006) could be detected. In contrast, a prospective study comprising more than 5000 healthy individuals could show that Y402H polymorphism was associated with an increased risk for myocardial infarction (Kardys *et al.* 2006), and supports the notion that factor H may have an important, protective role in atherogenesis. Genetic C4bp deficiency in humans is even more rare than factor H deficiency, and it associates with angioedema while no reports regarding atherosclerosis in these patients exist (Trapp *et al.* 1987).

1.1.4 C5b-9 is produced mainly via the alternative pathway

We found that the complement system had proceeded to its completion, i.e. to the generation of C5b-9, only in the deep musculoelastic layer of the intima, and that this was probably due to the absence of the complement regulators factor H and C4bp. However, the pathway - either classical or alternative - leading to the generation of C5b-9 in atherosclerotic lesions has not been fully clarified. Indeed, while no evidence of complement activation via the lectin pathway exist, various structures and molecules capable of activating either the classical or alternative pathway as well as signs of complement activation via these pathways have been detected in atherosclerotic lesions. In contrast, normal arterial intima lacks signs of complement activation, suggesting that complement-activating structures are either generated or deposited in the arterial wall during atherogenesis.

To investigate which pathway of complement activation was responsible for the generation of C5b-9 in the arterial intima, we compared the staining patterns of C4 and properdin, markers of classical and alternative pathway activation, respectively, with the staining pattern of the terminal membrane-attack complex (C5b-9) (Figure 4, in paper II). The superficial intima contained both C4c and properdin, suggesting that both the classical and alternative pathways had been activated in this area. In contrast, this area was devoid of C5b-9, likely reflecting the action of the complement inhibitors factor H and C4bp. Thus, only the deep musculoelastic layer contained C5b-9. Interestingly, this area was negative for C4c/d but strongly positive for properdin, suggesting that activation of the alternative pathway was responsible for the generation of C5b-9 in this area. Previous studies have shown that the deep intima contains enzymatically-modified LDL, which is known to activate the alternative pathway of complement (Torzewski *et al.* 1998b), hence rendering these modified LDL particles possible candidates for the observed activation of complement by the alternative pathway. We could

also demonstrate that, in the deep musculoelastic layer, C5b-9 does colocalize with cholesterol crystals, which are known to activate the alternative pathway of complement (Fig. 6, Oksjoki *et al.*, unpublished observation). In addition, it was found recently that immune complexes, which are traditionally regarded as activators of the classical complement pathway, also increase the activation of the alternative complement pathway by stabilizing C3 convertase (Ji *et al.* 2002). Thus, it is possible that immune complexes, which have been shown to be present in the arterial wall (Ylä-Herttuala *et al.* 1994), could participate in the generation of C5b-9.

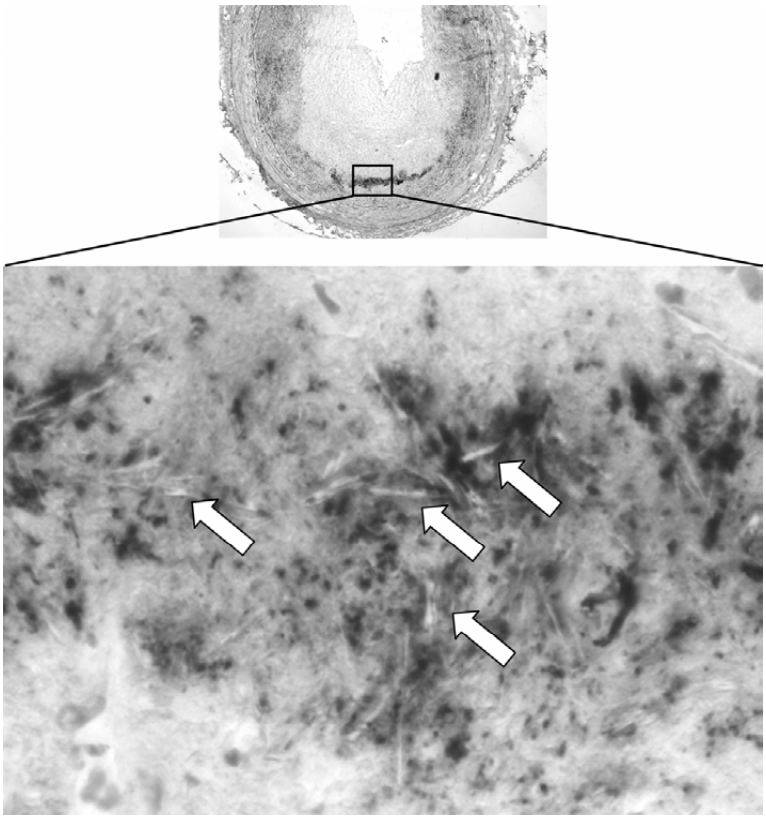

Figure 6. Complement terminal product C5b-9 (black) is deposited in the deep musculoelastic layer of the arterial wall, where it cololocalizes with extracellular cholesterol crystals (white clefts; marked with arrows).

Taken together, our findings suggest that activation of the alternative pathway is responsible for the generation of C5b-9 and thus, add to the current understanding of the mechanisms of complement activation in atherosclerotic lesions. In addition, our results support the hypothesis that, in atherosclerotic lesions, modified lipoproteins trigger complement activation leading to the generation of pro-inflammatory C5b-9.

1.2 Receptors for anaphylatoxin are expressed in atherosclerotic lesions (III)

Complement activation via any of the three pathways leads to the release of two potent anaphylatoxins, C3a and C5a. Both anaphylatoxins act on their target cells by binding and signaling through their ligand-specific receptors, which belong to the family of seven transmembrane domain G-protein-coupled receptors. Since complement is activated in atherosclerotic lesions, and anaphylatoxins are thus locally produced, we were interested to

learn whether the cells of human coronary arteries would express receptors for the two anaphylatoxins, C3a and C5a. Our immunohistochemical and PCR analyses revealed that these receptors, although almost absent from normal coronary intimas, were highly expressed in atherosclerotic lesions (Figure 1, in paper III).

To further characterize the cell types expressing these receptors in the lesions, we performed double immunofluorescence staining with antibodies against anaphylatoxin receptors and different cell types of the intima (Figures 2 and 3, in paper III). The major cell type expressing both C3aR and C5aR in the intima was the macrophage. Since intimal macrophages derive from circulating monocytes, we also investigated the expression of C3aR and C5aR during the maturation of human blood monocytes into macrophages in culture. We observed that freshly isolated monocytes expressed low but detectable levels of both C3aR and C5aR, and that during their maturation into macrophages, the expression of both receptors significantly increased, although the kinetics of the increase differed markedly between the two types of receptors. Our findings are consistent with the previous reports demonstrating that cells of monocyte–macrophage lineage express these receptors (Martin *et al.* 1997; Gerard & Gerard 1991), and that the expression of these receptors is increased after PMA-induced differentiation and activation of THP-1 cells (Gasque *et al.* 1998) and U937 cells (Crass *et al.* 1996) into macrophage-like cells. Regarding other inflammatory cells of the intima, the expression of C3aR and C5aR were more variable. Thus, while all T cells expressed C5aR, only about $1/10^{th}$ of them expressed C3aR. Previous reports have shown that circulating, unstimulated T cells express C5aR (Nataf *et al.* 1999), whereas activation of T cells is needed for them to express C3aR (Werfel *et al.* 2000). Thus, it is likely that the C3aR-positive T cells in the lesions are in an activated state. Moreover, all mast cells expressed C5aR, but none of them expressed C3aR. This lack of C3aR in mast cells was unexpected, since cells of the immature human mast cell line HMC-1 have been shown to express functional C3aR (Zwirner *et al.* 1998a) and moreover, human skin mast cells have been shown to respond to C3a by degranulation (Wuepper *et al.* 1972). However, no immunohistochemical reports on the presence of C3aR in human mast cells are available, and the functional effects of C3a on mast cells are assumed to be due to direct stimulation of G-proteins by this peptide (Mousli *et al.* 1992; Fukuoka & Hugli 1990). We also looked for the presence of C3aR in the mast cells of normal skin, inflamed intestinal wall and inflamed tonsils. All the mast cells in skin and intestine were negative, whereas a fraction of tonsillar mast cells were positive for C3aR. Hence, tissue-specific differences in the expression of C3aR in mast cells exist. Finally, in addition to inflammatory cells, both endothelial cells and superficial smooth muscle cells, but not those of deep intima or media, expressed C3aR and C5aR. This limited expression of anaphylatoxin receptors in smooth muscle cells may reflect the fact that a phenotypic switch from contractile to matrix-synthesizing cells is required for smooth muscle cells to be able to express these anaphylatoxin receptors.

Table 8. Summary of the expression of anaphylatoxin receptors by cells of human coronary atherosclerotic lesion

	macrophages	**T cells**	**mast cells**	**smooth muscle cells**	**endothelial cells**
C3aR	+	+ (about $1/10^{th}$)	-	superficial	+
C5aR	+	+	+	superficial	+

Signaling through anaphylatoxin receptors, especially C5aR, has been shown to mediate effects that potentially promote atherosclerosis and also affect the stability of the plaque. Thus, both anaphylatoxins may be involved in the recruitment of inflammatory cells into the intima by acting as chemokines (Arend *et al.* 1989; Zwirner *et al.* 1998b; Hartmann *et al.* 1997) and by upregulating the expression of adhesion molecules on endothelial cells (Foreman *et al.* 1996; Albrecht *et al.* 2004). In addition, both anaphylatoxins can induce activation and expression of pro-inflammatory cytokines by other intimal cells (Takabayashi *et al.* 1998; Takabayashi *et al.* 1996; Goodman *et al.* 1982; Ember *et al.* 1994; Albrecht *et al.* 2004; Monsinjon *et al.* 2003; Schulman *et al.* 1988; el-Lati *et al.* 1994). In addition, activation of mast cells and macrophages through C5aR has been shown to play a major causal role in triggering experimental immune complex-induced disease (Bozic *et al.* 1996; Hopken *et al.* 1997; Godau *et al.* 2004; Baumann *et al.* 2001; Shushakova *et al.* 2002), which may also be relevant for the progression of atherosclerosis, since immune complexes have been shown to be deposited in atherosclerotic intima (Ylä-Herttuala *et al.* 1994). Furthermore, previous studies have demonstrated that anaphylatoxins are spasmogenic to arteries (Vogt 1986). However, we found that smooth muscle cells of the contractile phenotype in the arterial wall, i.e. in the deep intima and the media, did not express anaphylatoxin receptors. Indeed, it has been suggested that spasmogenic effects by anaphylatoxins could actually be mediated only indirectly, i.e. by the vasoactive products released from macrophages and mast cells in response to their activation by anaphylatoxins (Marceau *et al.* 1990). Although C3a and C5a mostly promote similar responses, some opposing effects have also been reported. Thus, while C5a promotes adaptive immunity (Morgan *et al.* 1983) and drives Th1 type proatherogenic immune responses, C3a mediates immunosuppressive effects (Morgan *et al.* 1982) and drives Th2 type immune responses (Hawlisch *et al.* 2004), which, in turn, have been suggested to mediate antiatherogenic effects.

Taken together, we found anaphylatoxin receptors to be present in atherosclerotic lesions, in which they were concentrated in the shoulder areas containing the infiltrate of inflammatory cells. This novel finding implicates, that anaphylatoxins could participate in the activation of intimal cells and thus affect the inflammatory status, and possibly also stability, of the plaque. In support of this notion, high levels of circulating anaphylatoxins have been reported in patients with acute coronary syndromes compared to those with stable coronary artery disease (Kostner *et al.* 2005), and moreover, elevated plasma levels of C5a have been shown to independently predict future major adverse cardiovascular events in patients with established atherosclerosis (Speidl *et al.* 2005).

2 OxLDL-IgG immune complexes induce survival of human monocytes (IV)

One potential group of complement activators in atherosclerotic lesions include immune complexes, and a recent study verified that also autoimmune oxLDL-complexes are able to activate the complement via the classical pathway (Saad *et al.* 2006). OxLDL-IgG immune complexes have been detected in the circulation of humans, and lipoprotein-containing immune complexes have also been isolated from atherosclerotic lesions (Ylä-Herttuala *et al.* 1994). Regarding atherosclerosis, the role of such immune complexes is not fully clarified. It has been suggested that that the formation of these complexes could be involved in the removal of harmful oxLDL from the circulation via recognition of the complexes by phagocyte Fcγ receptors. However, a recent paper by Reardon and coworkers suggested these immune-mediated mechanisms do not seem to play a role in the clearance of modified LDL from plasma, at least in apoE$^{-/-}$ mice (Reardon *et al.* 2004). In contrast, it has been shown that lipoprotein-containing immune complexes can have many potentially proatherogenic effects on intimal cells. Thus, in addition to activating the complement, oxLDL-containing immune complexes have been shown, when added to cultured macrophages, to promote foam cell formation (Griffith *et al.* 1988; Lopes-Virella *et al.* 1997) and to induce the production of proinflammatory cytokines (Saad *et al.* 2006), oxygen radicals (Virella *et al.* 1995), and matrix metalloproteinases by macrophages (Huang *et al.* 2000). In addition, a prospective trial involving diabetic subjects suggested that high concentrations of LDL-ICs predicted the development of coronary artery disease (Lopes-Virella *et al.* 1999). In our study, we wanted to further investigate the potential roles of oxLDL-IgG immune complexes in atherogenesis. A previous study by Marsh *et al.* (Marsh *et al.* 1999) demonstrated that immobilized IgG molecules induced the production of monocyte colony-stimulating factor (M-CSF) by human monocytes. Accordingly, we hypothesized that oxLDL-IgG immune complexes (oxLDL-IgG ICs), in which IgG-molecules are bound to lipoprotein particles, could affect monocyte survival.

In our design of the study, we took into account the previous results suggesting that the size of immune complexes (ICs) is a critical factor in determining their biolocigal activity. Thus, previous studies have suggested that soluble (small) ICs, in contrast to immobilized ICs or insoluble (large) ICs, poorly cross-link Fcγ receptors and show decreased biologic activity (Jarvis *et al.* 1999). While circulating oxLDL-IgG ICs are mainly soluble, the arterial intima is likely also to contain immobilized oxLDL-IgG ICs, since at least part of oxLDL is matrix-bound via LPL (reviewed by Pentikäinen *et al.* 2002). Thus, to mimic such arterial immobilized ICs, we prepared ICs by 1) incubating rabbit IgG against human LDL together with oxLDL or 2) creating soluble ICs containing oxLDL and either human recombinant or serum derived anti-oxLDL antibodies, and bridging the formed soluble ICs together with $F(ab)_2$ fragments of goat anti-human IgG.

Our results showed that addition of oxLDL-IgG ICs to monocytes cultured in the absence of growth factors altered the morphology of monocytes and promoted their survival. Thus, monocytes cultured in the presence of oxLDL-IgG ICs remained attached to the surface of the well, were large, and spread along the surface of the well in contrast to the detachment and shrunken morphology of untreated control cells (Figure 1A, in paper IV). Further analysis by Oil Red staining and HPTLC revealed that oxLDL-IgG IC treatment transformed monocytes into foam cells (Figure II, online supplement, in paper IV). The uptake of complexes seemed to be mediated via the immunoglobulin part of the complexes, since the addition of oxLDL or acetyl LDL alone did not increase the content of intracellular cholesterol of monocytes. Thus,

the uptake of oxLDL in the form of immune complexes revealed a new mechanism, by which monocytes can already become transformed into foam cells at the stage when they do not express the scavenger receptors essential for the uptake of modified lipoproteins. This observation suggests that the process of foam cell formation may already begin early after the infiltration of blood monocytes into the subendothelial space of inflamed arterial intima. In addition to foam cell formation, the number of viable monocytes was significantly increased in response to oxLDL-IgG IC treatment (Figure 1B and Figure 6, in paper IV). Although previous studies had suggested that oxLDL alone could have survival-promoting effects in monocytes, we failed to detect such effects for oxLDL alone. Rather, our results, which demonstrated that immune complexes containing native LDL or KLH are able to induce similar increases in the number of viable monocytes, suggested that the survival-promoting effect of oxLDL-IgG ICs is mediated via IgG molecules (Figure 1C, in paper IV).

Regarding the increased viability of monocytes, we were interested to learn more about the mechanisms by which oxLDL-IgG ICs promote monocyte survival. First, we investigated whether the increased viability of monocytes in response to oxLDL-IgG IC treatment resulted from increased cellular proliferation or decreased apoptosis of cells. As demonstrated by the lack of incorporation of radioactive-labeled thymidine into nucleic acids, no proliferation in response to oxLDL-IgG ICs was observed. Rather, our results confirmed that oxLDL-IgG ICs protected monocytes from apoptotic cell death. Thus, following the addition of oxLDL-IgG ICs to monocytes, the amount of active caspase-3 and DNA fragmentation were reduced to levels comparable to cells cultured merely in the presence of growth factors, either M-CSF or GM-CSF (Figure 2, in paper IV). According to our original hypothesis, oxLDL-IgG ICs also induced a concentration-dependent increase in the production of M-CSF by monocytes (Figure 3A, in paper IV). Upon secretion, M-CSF may, in an auto- and paracrine manner, increase cellular survival by activating the PI 3-kinase-dependent pathway, resulting in the phosphorylation of Akt, a central survival factor in monocytes (Kelley *et al.* 1999). To our surprise, however, the production of M-CSF did not seem to be essential for the survival-promoting effects of oxLDL-IgG ICs. Thus, the neutralizing anti-M-CSF antibody did not affect IC-induced monocyte survival, while it efficiently blocked the effect of externally added M-CSF on monocyte survival (Figure 3B, in paper IV). Of interest, cross-linking of mouse Fcγ receptors (FcγRs) with monoclonal antibodies has also been shown to lead to PI 3-kinase activation and Akt phosphorylation in mice (Cao *et al.* 2004). Accordingly, we hypothesized that oxLDL-IgG immune complexes could mediate their effect on monocyte survival by direct binding to FcγRs with subsequent activation of Akt. Indeed, rapid phosphorylation of Akt after the addition of oxLDL-IgG ICs and the inhibition of monocyte survival by blocking the Akt-pathway strongly supported the direct activation of Akt via Fcγ receptors as the anti-apoptotic mechanism of immune complexes (Figure 4, in paper IV). Furthermore, experiments with receptor blocking antibodies revealed that the observed effect was mainly mediated by FcγRI (Figure 5, in paper IV). This is in line with the previous results showing the involvement of FcγRI and II in the uptake of and signaling induced by oxLDL-containing ICs in U936 and THP-1 cell lines and in human monocyte-derived macrophages (Huang *et al.* 2000; Lopes-Virella *et al.* 1997; Huang *et al.* 1999). Taken together, our findings reveal a novel mechanism by which oxLDL-IgG ICs can promote monocyte-macrophage accumulation in arterial intima, and thus, affect the pathogenesis of atherosclerosis.

Regarding atherosclerosis, previous studies have shown that apoptotic macrophages occur in atherosclerotic lesions, and that extensively oxidated LDL is strongly pro-apoptotic (reviewed by Martinet & Kockx 2001). In contrast, a very recent paper demonstrated that minimally

oxidized LDL (mmLDL), which was prepared by exposure of LDL to cells overexpressing 15-lipoxygenase, protected macrophages from apoptosis induced by both oxLDL and free cholesterol loading (Boullier *et al.* 2006). Of interest, the mmLDL-induced activation of PI3K/Akt-pathway and the blocking of this pathway completely eliminated the pro-survival effect of mmLDL. Thus, it is interesting to hypothesize that minimally modified LDL as well as oxLDL-IgG immune complexes could mediate survival-promoting effects in the lesions and thus to protect monocytes from the death-promoting effects of other factors, e.g. highly oxidized LDL.

What might be the implications of increased monocyte survival/apoptosis for the development of atherosclerosis? Obviously, increased monocyte survival tends to increase and maintain the intimal macrophage population. Various studies have suggested that, depending on the stage of the disease, macrophage survival/death may have opposing effects on atherogenesis and may be major determinants of lesion development (reviewed by Tabas 2005). To support the pro-atherogenic role of macrophages, previous studies have shown that apoE$^{-/-}$ mice that lack M-CSF, and therefore also lack tissue macrophages, show decreased atherosclerosis. In addition, deficiency of the p53 tumor-suppressor gene in macrophages, which leads to decreased macrophage apoptosis or increased proliferation, leads to increased lesion size in apo*3-Leiden mice (van Vlijmen *et al.* 2001) and LDLR$^{-/-}$ mice (Merched *et al.* 2003), respectively, and also causes the development of a more vulnerable lesion type. Similarly, lack of the proapoptotic protein Bax in macrophages, which leads to their decreased apoptosis, increases atherosclerosis in LDLR$^{-/-}$ mice (Liu *et al.* 2005). The authors of the study suggested that macrophage apoptosis provides a critical self-defence mechanism in suppressing atherosclerosis, since macrophage death may limit the number of inflammatory cells and thus also the local inflammation and synthesis of matrix metalloproteinases in the lesions. However, this may be true only if the apoptotic bodies of dying macrophages are cleaned efficiently by the neighboring phagocytes, which appears to be the case in early lesions. However, in more advanced atherosclerotic lesions, the uptake of apoptotic cells is impaired (Schrijvers *et al.* 2005), which may lead to secondary necrosis and the formation of a necrotic core and the accumulation of debris in intima. Thus, in early lesions, in which the uptake of apoptotic cells is still efficient, macrophage apoptosis may exert atheroprotective effects, whereas in advanced lesions with defective uptake, macrophage apoptosis might promote the generation of a necrotic core with a concomitant proinflammatory response (Tabas 2005).

3 Future perspectives

According to the current concepts, atherosclerosis is a chronic inflammatory state of the vessel wall with an autoimmune response against either foreign or altered self-structures present in the lesions. Despite the dramatic increase in the knowledge about the role of immune mechanisms in the pathogenesis of atherosclerosis during the past decades, our understanding of the complex role of the immune response in atherogenesis still remains rather fragmentary. Animal studies have established that activation of the immune response can have both protective (= anti-atherogenic) and adverse (= proatherogenic) effects in atherogenesis, and these effects may vary at different stages of the disease. However, elucidation of the role of specific parts of immunity could lead to insights into their potential roles as targets of therapeutic interventions. Thus, therapeutic interventions that specifically suppress the adverse, proatherogenic types of immunological responses or specifically strengthen the beneficial, anti-atherogenic types of responses could emerge as new ways of combating this disease. Although immune interventions in experimental animals have provided promising results, the relevance of these observations to human disease remains to be determined. When contemplating these issues, it is of special importance to keep in mind that immune responses have extremely important functions in the protection of the host against pathogens. Thus, it would be essential to be able to pharmacologically target the process of atherosclerosis without interference of the physiologically meaningful functions of the immune response. The following paragraphs discuss the role of complement system and immune response against plaque antigens, notably oxLDL, as possible therapeutic targets.

Regarding the complement system, experimental studies have suggested that complement activation, at least to its completion, is potentially proatherogenic. In addition, clinical human studies have suggested a correlation between increased levels of anaphylatoxins or C5b-9 and acute coronary events. It is therefore intriguing that that various inhibitors of the complement system and anaphylatoxin receptors have been recently generated. These drugs have already been tested in phase II-III human studies for the treatment of several inflammatory and autoimmune diseases, such as rheumatoid arthritis, asthma, psoriasis, nephritis, dermatomyositis, systemic lupus erythematosus, and paroxysmal nocturnal hematuria (Holland *et al.* 2004). In patients undergoing cardiopulmonary by-pass operation, the soluble complement receptor type 1 (Lazar *et al.* 2004) and the humanized monoclonal anti-C5 antibody (pexelizumab) (Verrier *et al.* 2004) have been shown to decrease mortality and the incidence of myocardial infarction. In addition, pexelizumab, which blocks the generation of both C5a and C5b-9, has already been used in clinical trials for acute myocardial infarction, and 24-h infusion was shown to significantly reduce 90-day mortality in patients undergoing angioplasty by attenuating the inflammatory response (Granger *et al.* 2003). Indeed, the effects of pexelizumab in conjunction with angioplasty in acute myocardial infarction are currently being studied in the APEX-AMI trial, which aims to recruit 8500 patients (Armstrong *et al.* 2005). It is also intriguing to hypothesize that orally administered anaphylatoxin-receptor antagonists could be used in the treatment of chronic inflammatory conditions such as atherosclerosis. In addition to having an easier route of administration, they have another advantage over the C5-inhibitor: they specifically block the interactions between C5a and C3a and their respective receptors and thus leave intact the other complement effector mechanisms necessary for immune protection.

During recent years, much attention has also been paid to the possibility to use the immune response against oxLDL as a therapeutic target. Thus, the fascinating idea of the possibility to

vaccinate against atherosclerosis has risen from several studies, which have provided evidence that immunization with atherosclerosis-related antigens such as oxLDL can affect atherogenesis in experimental animals. Indeed, in mouse models of atherosclerosis, parenteral immunization with preparations of oxidized LDL has been shown to evoke an atheroprotective immune response and thus, reduce the extent of the plaques up to 40-70%. To further characterize the epitopes leading to protective immunity, Fredrikson *et al.* recently demonstrated that some of the 20-amino acid peptide sequences of apoB-100 (both native and MDA-modified), against which humans have been shown to developed immune response, also reduced the extent of atherosclerotic lesions in a mouse model when administered in the vaccine formulation (Fredrikson *et al.* 2003b; Fredrikson *et al.* 2005). In addition to decreasing the lesion size, immunization also led to a more stable plaque phenotype as reflected by the decrease in the number of macrophages and increase in the content of collagen. Furthermore, the immune response was characterized by a shift towards antiatherogenic Th2-type of immune response as suggested by the significant increase in the Th2-type antibody IgG_1. Interestingly, also passive immunization with human IgG_1 generated against MDA peptide sequences reduced atherosclerosis in apoE-/- mice (Schiopu *et al.* 2004). In addition to apoB-100 peptides, also phosphorylcholine present in oxLDL has been suggested to provoke an atheroprotective immune response, which is characterized by the generation of IgM natural antibodies. As phosphorylcholine is part of the *S. pneumoniae* cell wall, Binder *et al.* hypothesized and could show that immunization with *S. pneumoniae* induced an increase in phosphorylcholine antibodies, which, likely by cross-reacting with oxLDL, reduce atherosclerosis in $LDLR^{-/-}$ mice (Binder *et al.* 2003). These promising results have generated optimism concerning vaccination with epitopes of oxLDL as a future therapeutic option in the combat against atherosclerosis. However, before applying this strategy to humans, various issues need to be clarified, i.e. the key antigenic determinants involved in atherosclerosis, the most suitable adjuvant and route of administration, and the safety and durability of the effect. In contrast to oxLDL, immunological tolerance against HSP60/65 and β2GPI through activation of mucosal immunity has been shown to lead to decreased atherosclerosis in animal models (Maron *et al.* 2002; George *et al.* 2004). This atheroprotective effect is likely to involve the activation of regulatory T cells. Similarly, induction of mucosal tolerance against disease-related antigens has been shown to be efficient in decreasing autoimmune diseases in experimental animals as well as in humans (Faria & Weiner 2005). Thus, approaches based on the induction of oral tolerance for disease-related antigens could also emerge as a new immunomodulatory therapy for atherosclerosis.

VI SUMMARY AND CONCLUSIONS

Current evidence indicates that immune mechanisms play a role in atherogenesis. The purpose of this study was to examine the activation and regulation of one immune mechanism, the complement system, in atherosclerotic lesions, and to investigate the effects of oxLDL-containing immune complexes on the survival of cultured human monocytes. Based on our results, the following summary and conclusions can be presented:

1. The regulators of the alternative and classical pathways of the complement system, factor H and C4bp, respectively, were present in the human coronary arterial intima during all stages of atherosclerosis. In early to intermediate lesions, these inhibitors were located in the superficial intima, where they colocalized with arterial proteoglycans. In addition, both factor H and C4bp were able to bind to human aortic proteoglycans *in vitro*.

2. Immunohistochemical evidence suggested that, in the superficial proteoglycan-rich layer of the intima, which contained factor H and C4bp, complement activation was limited to the C3-level. Thus, although this area contained both activators of the complement system (IgM, CRP) and also markers of activation via the classical (C4c/d) and alternative pathways (C3d, properdin), no terminal C5b-9 was present in the area. In contrast, C5b-9 was present in the deep musculoelastic layer of the intima and showed very little overlap with factor H and C4bp.

3. Since the results of immunohistochemistry suggested that the proteoglycan-rich layer of the coronary intima was protected from full-blown complement activation, we tested for the ability of arterial proteoglycans to regulate complement activation *in vitro*. We found that both arterial proteoglycans and heparin glycosaminoglycan were able to inhibit complement activation in human serum. The results suggested to us that, in the superficial intima, proteoglycans bind the complement inhibitors C4bp and factor H and thus, may block the complement activation from proceeding to the terminal level.

4. In the musculoelastic layer of the intima, possibly due to the absence of regulation, complement activation proceeded to the assembly of C5b-9. Our results, which demonstrated the presence of properdin, but not C4c/d, in this area suggested that C5b-9 was generated as a result of activation of the alternative pathway of the complement.

5. Complement activation generates two potent anaphylatoxins, C3a and C5a. Here we showed that, in contrast to normal intima, the cells of atherosclerotic lesions expressed the anaphylatoxin receptors C3aR and C5aR and thus had the potential to respond to anaphylatoxins. The major cell type expressing these receptors in the lesions was the macrophage. In addition, endothelial cells and superficial smooth muscle cells expressed both C3aR and C5aR, all T cells expressed C5aR, and a small fraction of them also expressed C3aR, whereas mast cells only expressed C5aR. These findings suggest that anaphylatoxins can play a role in atherogenesis and its complications.

6. During atherogenesis, lipoprotein-containing immune complexes are deposited in the arterial wall and able to activate the classical pathway of the complement. We were able to show that oxLDL-IgG immune complexes induced survival of human monocytes by decreasing their spontaneous apoptosis. This effect was mediated via crosslinking of the Fcγ receptor I with subsequent activation of the Akt-dependent signaling pathway. In addition, the

oxLDL-IgG immune complexes transformed human monocytes into foam cells, and this already occurred at the stage when the expression of scavenger receptors and, thus, the uptake of modified lipoproteins alone are known to be very low. Modulation of monocyte survival may affect the genesis and maintenance of the intimal macrophage population, which, based on previous observation, may promote atherosclerosis at least in the early stages of the disease.

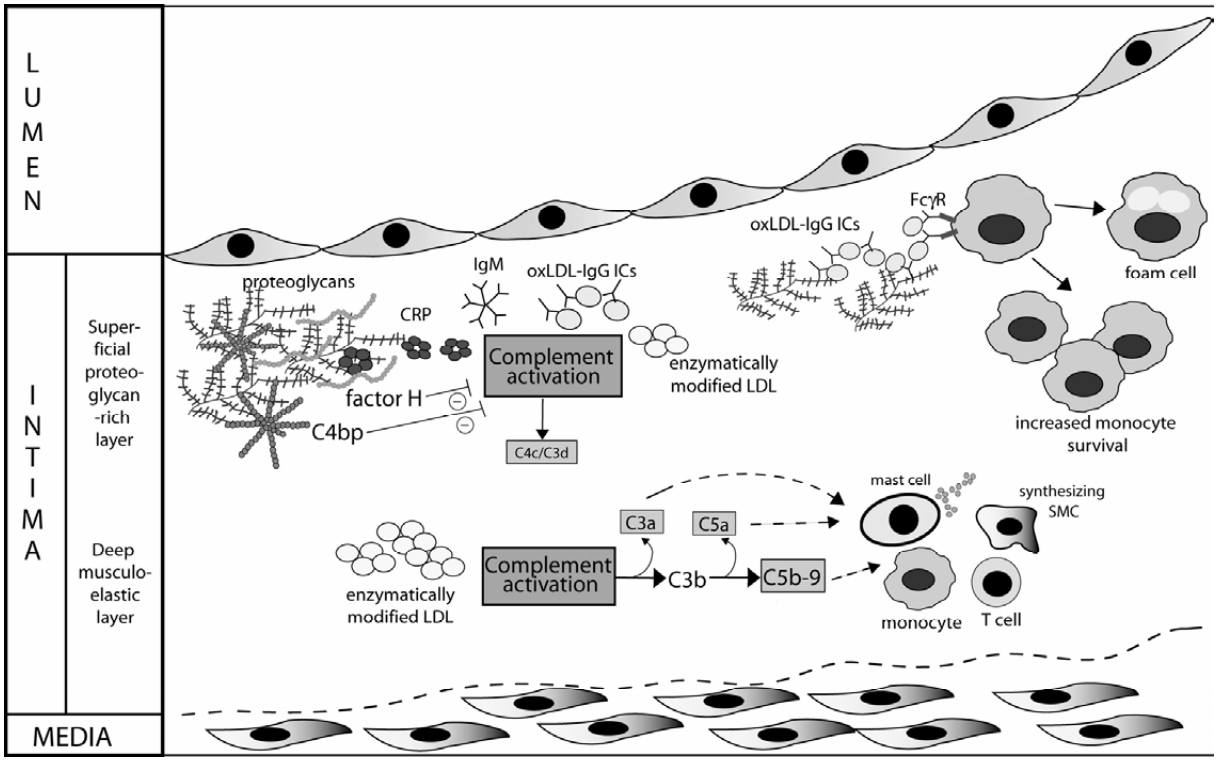

Fig. 7. Summary of the results. We found that, in the superficial proteoglycan-rich layer, both the classical and alternative complement pathways had been activated, possibly via CRP, immune complexes, and modified lipoproteins. However, in this area, complement activation had been restricted to the C4c and C3d –levels, suggesting that complement activation was regulated. Indeed, regulators of the classical and alternative pathways, C4bp and factor H, respectively, were present in this specific area and bound to arterial proteoglycans *in vitro*. In addition, arterial proteoglycans could inhibit the activation of complement *in vitro*, an effect mediated probably via their interaction with complement inhibitors. Deeper in the intima, without C4bp and factor H, complement activation had proceeded to the terminal C5b-9-level via activation of the alternative pathway. In this area, C5b-9 may exert proinflammatory effects on various intimal cells. In addition, we detected expression of the receptors for complement-derived anaphylatoxins on intimal cells. Finally, we found that oxLDL-IgG ICs, in addition to transforming monocytes into foam cells, promoted their survival by decreasing their spontaneous apoptosis in an FcγR-dependent manner.

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